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An investigation into the effects of circadian rhythm on the efficacy of sibutramine and d-fenfluramine, two centrally-acting anorectic agents: focussing on the impact on the enhancement of satiety

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A thesis submitted for the degree of Doctor of Philosophy

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Contents

1.0 Abstract

This thesis is concerned with the impact of circadian rhythmicity upon the effect of two centrally acting anti-obesity agents, sibutramine and *d*-fenfluramine, in the behavioural satiety sequence (BSS) and their impact on locomotor activity. Experiments were carried out at 6 timepoints evenly spread over the circadian cycle and the results compared to provide a measure of the effect of circadian rhythm on drug efficacy and action. The BSS was exhibited and recorded at all times except late in the light phase when animals showed no interest in feeding regardless of drug treatment. Using a time-sampling methodology in animals subjected to 6-hour food deprivation both sibutramine (1.67 mg/kg) and *d*-fenfluramine (1.0 mg/kg) produced hypophagic effects through the enhancement of satiety, at all circadian times except late in the light phases when there was no feeding response under control conditions. Both drugs showed greater potency for satiety enhancement during the dark phase when natural feeding is more prevalent. Drug treatment accelerated the offset of feeding and the onset of resting (promoting total observations of resting and suppressing those of feeding) to greater degree in the dark phase, obscuring the circadian changes in behaviour seen under control conditions. Both drugs reduced food intake at all points in the dark phase and early in the light phase. At mid-light phase *d*-fenfluramine decreased food intake but sibutramine did not. In locomotor activity studies using the same doses, food deprivation, food presentation paradigms and circadian timepoints as the BSS studies, sibutramine decreased activity in a manner consistent with the enhancement of satiety when pooling data from all 6 timepoints. In contrast *d*-fenfluramine had different effects on motor activity at mid-light and mid-dark phase but no overall impact. At mid-light phase *d*-fenfluramine caused an initial depression of activity but the animals were more active over the second half of the experiment; this pattern was not seen in the dark phase. This discrepancy could explain previous reports of *d*-fenfluramine disrupting the BSS in experiments carried out in the mid light-phase, although results described here suggest the BSS is preserved at mid-light phase. Neither drug significantly modified exploratory locomotion, an indicator of sedation, at the doses used for the circadian locomotor activity or BSS studies. The present results suggest that circadian rhythms have an influence over the response to sibutramine and *d*-fenfluramine: tests with these drugs should be targeted to the dark phase for maximal effect and relevance.

2.0 Abbreviations

5-HT	–	5-Hydroxytryptamine; Serotonin
AgRP	–	Agouti-related protein
ANOVA	–	Analysis of Variance
ARC	–	Arcuate nucleus (of the hypothalamus)
BSS	–	Behavioural Satiety Sequence
CART	–	Cocaine- and amphetamine-regulated transcript
CCK	–	Cholecystokinin
CNS	–	Central Nervous System
CT	–	Circadian Time
DOI	–	1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane
ED_{50}	–	Effective Dose 50; the dose which produces a 50% response
GMT	–	Greenwich Mean Time
i.p.	–	Intra-peritoneal
LHA	–	Lateral Hypothalamic Area
*m*CPP	–	1-(*m*-chlorophenyl)piperazine
MDMA	–	3,4-methylenedioxy-metamphetamine; ecstasy
MSH	–	Melanocyte Stimulating Hormone
NA	–	Noradrenaline
NPY	–	Neuropeptide Y
NTS	–	Nucleus of the Solitary Tract
POMC	–	Pro-opiomelanocortin
PVN	–	Paraventricular Nucleus
RBI	–	Research Biochemicals International
SCN	–	Suprachiasmatic Nucleus
SNRI	–	Serotonin-Noradrenaline Re-uptake Inhibitor
SSRI	–	Selective Serotonin Re-uptake Inhibitor
VMH	–	Ventromedial Hypothalamus

3.0 Introduction

3.1 A Brief Overview of the Control of Appetite and Food Intake

Obesity has become a worldwide problem of epidemic proportions (*e.g.* James *et al.*, 2001). The study of appetite, feeding and satiety has therefore become a major subject of research, with the dual aims of understanding how such processes are controlled and investigating possible pharmacotherapies to aid the obese in losing weight. This thesis is based on work done to extend knowledge about the centrally-acting anti-obesity drugs sibutramine and *d*-fenfluramine, specifically with regard to how circadian rhythm affects the efficacy of these agents. A brief look at the control of feeding is an appropriate beginning.

In the Western World we live in an environment where food is never scarce and famine is something that happens to other people. It was not always so, and in an evolutionary sense this is a recent development, one which appears to have led to a breakdown in the homeostatic regulation of bodyweight and an ever more obese population. So what are the systems that control appetite and food intake, and how are they disrupted in obesity?

There are both tonic and episodic elements to the control of appetite and feeding, released from a variety of tissues and in response to different stimuli. Perhaps the most closely studied of the factors is leptin, a cytokine hormone synthesised and released from adipose tissue, which serves as a tonic indicator of adiposity and acts to depress appetite, but there are a multitude of other factors at work, including insulin, galanin, cholecystokinin, orexins and more.

Tonic Regulation. Tonic regulation is achieved primarily through leptin and insulin, released from adipocytes and the pancreas respectively. Leptin is released in relation to the degree of fat stores in the adipocytes, with fat-laden cells releasing more leptin than those with less stored. Insulin release is stimulated by blood sugar levels, so the two primary tonic signals are responsive to different macronutrient stores, although insulin is also responsive to adiposity as blood glucose levels tend to be elevated in obesity (Arch, 2005). Both these tonic signal molecules are released in the periphery

and are relayed to the central nervous system, where they signal for a reduction in food intake. Conversely, a period of restricted food intake leads to a decline in leptin and insulin levels (Wynne *et al.*, 2005). Leptin and insulin are both capable of increasing energy expenditure too. But how do these signals bring about their responses?

Leptin receptors are widespread in the hypothalamus and the brainstem: the former being a well characterised centre of homeostatic regulation with a prominent role in the control of food intake and with strong interconnections with areas of the latter. Leptin is actively transported across the blood-brain barrier, a transport which is decreased under conditions of starvation (Wynne *et al.*, 2005) but also in obesity (Arch, 2005); once across leptin activates receptors in various different nuclei. However, although there are leptin receptors in many areas that are protected by the blood-brain barrier, perhaps the primary sites of action are in the arcuate nucleus of the hypothalamus (ARC) and the nucleus of the solitary tract (NTS) in the brainstem, two structures that are situated in close proximity to sites where the blood-brain barrier is incomplete – the median eminence and the area postrema respectively. The ARC is considered to be central to the integration of appetitive signalling (Wynne *et al.*, 2005) and has extensive interconnections with both other nuclei of the hypothalamus such as the ventromedial hypothalamic nucleus (VMH), lateral hypothalamic nucleus (LHA) and the paraventricular nucleus (PVN) as well as with the NTS in the brainstem (Wynne *et al.*, 2005; Berthoud, 2002). All of these areas express leptin receptors and may be targets for endogenous leptin; administration of exogenous leptin directly into the fourth ventricle or over-expression of leptin in the hypothalamus both reduce food intake. Additionally these nuclei all have further roles in the control of feeding and appetite, with neuronal circuits interlinking them extensively.

These neuronal circuits include neurons which express the leptin receptor – a single transmembrane cytokine receptor – and signal through neuropeptides. The ARC is a point of integration between these neuropeptide signals and circulating peripheral signals (Wynne *et al.*, 2005; Bethoud 2002). It contains two primary cell populations – one which stimulates feeding and one which inhibits food intake (Wynne *et al.* 2005; Bethoud 2002). The feeding-inhibitory neurons express pro-opiomelanocortin

(POMC), a pre-cursor protein for melanocortins and melanocye stimulating hormones (MSHs), and cocaine- and amphetamine-regulated transcript (CART). The other population of cells express neuropeptide Y (NPY) and agouti-related protein (AgRP), two mediators that both stimulate feeding – NPY by agonistic action at postsynaptic receptors, and AgRP by antagonising α-MSH, a POMC cleavage product (Wynne *et al.*, 2005). Both populations express receptors for leptin, which activates the POMC/CART-expressing cells and inhibits the activity of NPY/AgRP cells. Thus, in principle, when adiposity, and thus leptin levels, is high in times of plenty the orexigenic neurones are inhibited whilst the populations that inhibit feeding are activated. In times of starvation when leptin levels are lower the NPY/AgRP neurons are freed of inhibition and the POMC/CART neurons lack stimulation, so the orexigenic response dominates.

But if leptin inhibits feeding and is raised in times of high adiposity, why is obesity not prevented or counteracted by the increased leptin that fat laden adipocytes produce? Part of the issue is believed to be the decreased brain penetration by leptin in obesity and the phenomenon of secondary resistance – where persistent high levels of leptin result in desensitisation of leptin receptors– in a parallel to mechanisms seen with other signalling molecules. However it is also possible that it is not true resistance and simply a result of leptin not being a sufficient mechanism to cope with the modern lifestyle and environment (Arch, 2005).

Returning to the neuropeptide circuits, both populations of neurons project into other hypothalamic nuclei in tandem – such as the PVN, where elevation of NPY (endogenous or exogenous) stimulates feeding and conversely α-MSH (an endogenous neuropeptide arising from cleavage of POMC) inhibits food intake (Wynne *et al.*, 2005). The PVN is also responsive to many other episodic mediators affecting food intake – including the monoamines serotonin and noradrenaline [section 3.2, below], and insulin.

Insulin, like leptin, is a peripherally-produced hormone with widespread receptors in the hypothalamus – including the ARC and PVN. Activation of these receptors can both prevent starvation-induced rises in NPY and increase the expression of the

POMC mRNA (Wynne *et al.*, 2005). Thus insulin can act as a tonic regulator of feeding, and does so through similar neuropeptide pathways as leptin.

Episodic Regulators. Together with the tonic factors there are a variety of short-term, episodic, signals involved in the control of appetite and food intake. There are too many to cover them all in depth, with different factors released from different parts of the body in response to different stimuli – some that stimulate feeding and some that inhibit food intake. Some of these signals are released into the peripheral circulation (*e.g.* ghrelin), whilst others are relayed to the brain by the vagus nerve (*e.g.* peripheral cholecystokinin signals), and some – like the orexins – are themselves neuropeptides released when neurons detect changing metabolite levels.

Ghrelin is an example of an orexigenic peripheral factor. Released into the circulation, it is primarily produced by cells in the stomach, but also to a lesser degree from populations lower down the gut. Plasma levels are high in times of fasting, and low after ingestion of energy (food or glucose solution) but not after ingestion of water alone – so ghrelin release is not simply regulated by gastric distension. Peripheral ghrelin is thought to produce its orexigenic effect via the activation of NPY neurons in the ARC (Wynne *et al.*, 2005) but ghrelin is also expressed centrally by some hypothalamic neurons with projections to various nuclei, such as the PVN or LHA which are implicated in the control of feeding, where they act on populations expressing both POMC/CART and NPY/AgRP. They also terminate on orexin neurons in the LHA.

Cholecystokinin (CCK) is a gut satiety factor predominantly expressed in duodenum and jejunum and expressed in the aftermath of ingesting nutrients. It has many actions to do with co-ordinating digestion of food, but is also capable of directly inhibiting food intake. Peripheral CCK signals activate the vagal nerve, which projects to the NTS in the brainstem, and the signal is then forwarded from the NTS to the hypothalamus (Wynne *et al.*, 2005). The NTS contains many POMC/CART neuron cell bodies which are activated by peripheral CCK. Although this is the primary pathway for CCK signalling, it is also possible that circulating CCK is transported across the blood-brain barrier and directly into the central nervous system. It is also

true that CCK is widely expressed in the brain, where it functions as a neuropeptide and is involved in many and varied neural pathways – including satiety circuits.

A pattern is clearly visible with the examples given here: whether tonic or episodic, conducive or inhibitive of feeding, the factors involved in appetite control exert their effects by influencing the neural circuits in the hypothalamus, activating either POMC/CART neurons or NPY/AgRP neurons.

But what of the orexins – which are themselves peptide neurotransmitters that stimulate feeding? In contrast to ghrelin, CCK, leptin and insulin which lie upstream of the hypothalamic circuits described above, orexin may be downstream of NPY, with the orexin cell bodies in the LHA expressing receptors for NPY (and leptin). Additionally, orexin neurons may be responsive to circulating glucose levels – certainly there are glucose-sensitive cells in the LHA – and thus it is possible that dips in blood glucose may help initiate feeding behaviour (Arch, 2005). Orexin neurons project widely to other regions of the hypothalamus as well as to structures involved in arousal and motor control, but what lies downstream of orexin systems is not yet fully clear (Wynne *et al.*, 2005).

The above should give a little background on the control of feeding and appetite (although far from an exhaustive description). However this thesis is concerned with drugs which exert their effects by acting on monoamine neurotransmitter systems and so a discussion of these circuits and how they relate to feeding control follows.

3.2 The Role of Serotonin and Noradrenaline

This section concentrates on giving an overview of the role of the monoamines noradrenaline (NA; **figure 3.6.1**) and serotonin (5-hydroxytryptamine, 5-HT; **figure 3.6.2**) in the control of feeding behaviour as these are the neurotransmitters upon which sibutramine and *d*-fenfluramine predominantly act. Many in-depth reviews of the processes involved with feeding and satiety have been written over the last 20 years (*e.g.* Blundell and Halford, 1999; Blundell, 1986; Hoebel, 1997; Leibowitz and Alexander, 1998; Leibowitz and Hoebel, 1998; Leibowitz and Shor-Posner, 1986; Simansky, 1996) and the reader is encouraged to look to these for further and more complete understanding of the regulation of food intake and feeding behaviour.

Both NA and 5-HT are heavily implicated in general behavioural arousal. Equally both have been proven to have specific actions in regulating feeding behaviour (NA and 5-HT) and satiation and satiety (5-HT), the process of attaining and maintaining a state of "fullness" that accompanies the natural termination of feeding. The hypothalamus – and in particular the paraventricular nucleus (PVN) – is considered to be the region of the brain most central to the control of feeding behaviour and food intake though other regions are undoubtedly involved (*e.g.* Leibowitz and Hoebel, 1998).

Both α_1 and α_2 adrenoreceptors have a role in modulating feeding in the hypothalamus, and specifically within the PVN (Goldman *et al.*, 1985; Jhanwar-Uniyal *et al.*, 1986; Leibowitz and Hoebel, 1998; Morien *et al.*, 1999; Wellman *et al.*, 1993). It has been shown that whilst α_1 receptor activation leads to the cessation of feeding and a reduction in food intake, agonists at α_2 receptors actually increase food intake and stimulate a feeding response in satiated animals (Goldman *et al.*, 1985; Jhanwar-Uniyal *et al.*, 1986; Leibowitz and Hoebel, 1998; Wellman *et al.*, 1993). *In vivo* NA acts preferentially through α_2 receptors over α_1 receptors (Wellman *et al.*, 1993), hence the specific increase in food consumption seen (on top of general increases in arousal) if NA is administered into the PVN. The balance of α_1 and α_2 receptors is thought to play a role in the extent of this.

The interaction between the two receptor types is not only directly antagonistic, but also linked in terms of receptor expression; the time of peak expression for one subtype corresponding with the nadir of expression for the other. Binding studies on tissue harvested at times spread across the circadian cycle found expression of α_1 receptors to be highest in the light phase and lower during the dark phase (Morien *et al.*, 1999). The opposite was true of α_2 receptors (Jhanwar-Uniyal *et al.*, 1986; Morien *et al.*, 1999); thus receptors that stimulated feeding exhibited greater expression in the PVN at times of peak food and water intake. Studies with selective agonists and antagonists have demonstrated that these two receptor subtypes can be in direct competition; Goldman *et al.* (1985) showed that administration of the α_1 antagonist corynathine enhanced the feeding response induced by intra-PVN injections of NA. The interaction between the levels of α_1 and α_2 receptors is linked to circadian time and is just one reason why circadian rhythm [section 3.3, below] may be an important factor in relation to drug treatment of obesity, with NA re-uptake inhibition being a crucial part of the mechanism of action of sibutramine [see section 3.4 for a review of sibutramine].

The role played by NA seems small compared to the impact of 5-HT on food intake, feeding and satiation. This latter neurotransmitter is very prevalent in the hypothalamus, where it has roles in two systems central to this thesis. First, modifications of 5-HT can cause modulations in the suprachiasmatic nucleus (SCN) – the brain region at the centre of the circadian clock functions [section 3.3], and not entirely dissociated from feeding (Leibowitz and Alexander, 1998) – and secondly 5-HT plays a large part in regulating food intake, with specific reference to the processes of satiation and satiety (Halford and Blundell, 1999; Leibowitz and Alexander, 1998; Simansky, 1996). Serotonin plays a role in modulating satiety, both in the periphery and within the central nervous system (*e.g.* Edwards and Stevens, 1991; Simansky, 1996). It is considered to be a short-term satiety factor, working in conjunction with other modulators such as cholecystokinin (CCK; Cooper *et al.*, 1990*a*; Leibowitz and Hoebel, 1998) – which itself is a peripheral satiety factor (*e.g.* Antin *et al.*, 1975) – yet whilst the 5-HT releasing agent fenfluramine produced anorexia dependent on CCK (Cooper *et al.*, 1990*a*) the anorectic effect of the 5-HT re-uptake inhibitor fluoxetine did not share this dependence (Cooper *et al.*, 1990*b*). Many drugs that enhance 5-HT neurotransmission – such as sibutramine [section 3.4]

and fenfluramine [section 3.5], the two drugs with which this thesis is concerned – exert actions on feeding behaviour and food intake by facilitating or speeding up the natural processes of satiation and satiety, as measured by the Behavioural Satiety Sequence (BSS; Antin *et al.*, 1975; Halford *et al.*, 1995; Halford *et al.*, 1998; chapters 4 and 7). This is to say that the normal behavioural structure of feeding and meal termination is preserved but temporally advanced, mimicking the effect of "pre-feeding." Serotonin exerts its actions through a large number of different receptor types (see Barnes and Sharp (1999) for a review of 5-HT receptors), not all of which are strongly linked to appetite, feeding or satiety. Of primary importance to these functions are the 5-HT_{1B} and 5-HT_{2C} subtypes, which play specific roles in determining food intake (5-HT_{1B}) and the rate of eating (5-HT_{2C}) respectively (Grignaschi and Samanin 1992; see Clifton (2000), Simansky (1996) and Leibowitz and Alexander (1998) for reviews). Activation of the 5-HT_{1A} receptor has been shown to elicit hyperphagia (Hutson *et al.*, 1988; Simansky, 1996). These roles have been determined by studies with specific agonists such as CP-94,253 (a 5-HT_{1B} receptor agonist; Halford and Blundell, 1996*a*; Lee and Simansky, 1997; Lee *et al.*, 2002) and using specific antagonists, such as ritanserin, cyanopindolol and metergoline, to investigate the contributions of various receptor subtypes to fenfluramine hypophagia (Grignaschi and Samanin, 1992). Additionally, in the case of the 5-HT_{2C} receptor experiments have been done using knockout mice (Tecott *et al.*, 1995; Vickers *et al.*, 1999); animals lacking this receptor developed obesity in adulthood and exhibited less of an anorectic response to fenfluramine, argued to be a result of a reduced satiating effect (Vickers *et al.*, 1999). Selective agonists at 5-HT_{1B} receptors such as CP-94,253 tend to enhance satiety, as defined by advancing and preserving the BSS (Halford and Blundell, 1996*a*; Lee and Simansky, 1997; Lee *et al.*, 2002; see section 4.1). Agonists acting at 5-HT_{2C} receptors have produced mixed results with some producing disruption of the BSS through sedation, at least at high doses (*e.g.* MK-212; Halford *et al.*, 1996), whilst others appear to advance and preserve the BSS (e.g. *m*CPP (1-(*m*-chlorophenyl)piperazine) and Ro 60-0175 ((*S*)-2-(6-chloro-5-fluoro-indol-l-yl)-l-methylethylamine hydrochloride); Hewitt *et al.*, 2002). All these drugs reduced food intake, however.

Activation of other 5-HT receptor subtypes can influence food intake and feeding, too, though without specifically affecting satiety and satiation. For example as mentioned

above, activation of the 5-HT_{1A} receptor can cause hyperphagia (Hutson *et al.*, 1988; Simansky, 1996). This is related to this receptor's primary function as a cell body autoreceptor on 5-HT neurones, including those projecting to the hypothalamus; activation of these inhibitory autoreceptors decreases neuronal firing rates and consequently reduces neuronal 5-HT release, thus inhibiting 5-HT mediation of satiety. In contrast the 5-HT_2 receptor agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) reduces food intake, but does so by facilitating a general increase in activity (Kitchener and Dourish, 1994), reminiscent of the effect of dopaminergic agents like amphetamines, which disrupt natural satiety (*e.g.* Halford *et al.*, 1998).

As with NA, the PVN is thought to be the primary site of action for 5-HT effects on feeding behaviour (*e.g.* Simansky, 1996; Leibowitz and Alexander, 1998) but its effects are not limited to those produced in this nucleus. Lesions of the PVN fail to prevent the modification of feeding by some serotonergic agents (Fletcher *et al.*, 1993) and other nuclei – including the SCN – are thought to play a role (*e.g.* Simansky, 1996; Leibowitz and Alexander, 1998). Both 5-HT_{1B} and 5-HT_{2C} receptors are expressed in high numbers in the hypothalamus as a whole, and specifically in the PVN and related structures like the SCN, whilst 5-HT_{1A} receptors are scarce in these regions (Leibowitz and Alexander, 1998).

So how do these monoamine neurotransmitters fit in with respect to the neuronal peptide circuits mentioned in section 3.1? Are the two systems separate or do they interact with each other? The answer is intuitive: the systems must interact somehow, and they do. Neurons expressing the feeding-inhibitory 5-HT_{1B} receptor are not just found in the PVN, but also in the ARC, the LHA and other feeding-associated nuclei. At least in the PVN and LHA this pattern of expression is consistent with populations of NPY neurons – thus the inhibitory action of 5-HT on feeding could in part be down to a suppression of NPY activity. Moreover, activation of PVN 5-$HT_{2A/2C}$ receptors can also antagonise NPY-induced increases in feeding (Ramos *et al.*, 2005). It is also known that, when given peripherally, the 5-HT releasing agent *d*-fenfluramine [section 3.5, below] can decrease NPY concentrations in several hypothalamic nuclei (Ramos *et al.*, 2005). NPY and noradrenaline can be found in the same neurons in some areas of the brain and NPY can also stimulate NA release. The mechanisms and

role of NPY in modulating noradrenergic systems are not fully elucidated yet (Ramos *et al.*, 2005). Less still is clear about interactions between POMC-cleavage products such as α-MSH and monoamines, but it has been shown that blocking the receptors through which α-MSH acts can lessen the anorectic response to the serotonin releasing agent *d*-fenfluramine (Heisler *et al.*, 2002; Ramos *et al.*, 2005).

The above is a focussed summary of some actions of 5-HT and NA in the control of feeding and satiety. There are many other roles played by both these neurotransmitters in fields related to appetite, obesity and satiation – including impact on dietary selection of macronutrients for example – and by other compounds such as the peptides CCK and neuropeptide Y, or hormones such as leptin. Rather than paint the complete picture of the neuroscience of feeding behaviour, the aim here was to provide an insight into how NA and 5-HT modulations can affect feeding behaviour and why drugs that work through these systems may show efficacy in treating obesity. For further understanding of the roles played by other modulators the reader is advised to consult Leibowitz and Hoebel (1998).

The above overview also highlighted one reason why circadian rhythm is an important factor to consider – namely the circadian variation in PVN α-adrenergic receptor expression and the balance between the two subtypes. This is not the only factor that varies with circadian time, whether related to the interference with the control of feeding specifically or drug treatment in general, and the next section will give an overview of why circadian rhythm is an important factor to consider in pharmacology.

3.3 The Importance of Circadian Rhythm

Mammalian organisms exhibit rhythmic changes in a number of biological parameters with time of day. These 24-hour rhythms are driven by an endogenous pacemaker located in the SCN (see Weaver, 1998 for a single review article or the special issue of *Chronobiology International* (**15**(5), 1998) for a collection of in depth articles on the SCN), and also influenced by environmental cues such as those provided by light. The circadian rhythm is an endogenous rhythm. Isolating an organism from external cues does not eliminate rhythmicity (Rae, 1998), thus proving the endogenous component. Moreover, the endogenous rhythms – such as that shown in the level of activity – "free run" in the absence of external cues (Rae, 1998); rather than the periods of activity occurring at the same time every day, they occur with a periodicity of just over or just under 24 hours. It is from here that the term "circadian rhythm" comes (Latin: *circa* (around) and *dies* (day) and hence literal meaning of circadian is "around a day"). In the presence of a regular light cycle, and the photic cues this provides, these endogenous circadian rhythms become entrained to the 24-hour photoperiod (*e.g.* Bunning, 1964; Davies *et al.*, 1972; Rae, 1998). Since the driving of the rhythmicity is at least partially endogenous, this entrainment by external factors affects most, if not all, of the biological systems that exhibit rhythmicity.

Circadian rhythmicity is expressed in the variation in biological parameters with time of day. Many different systems exhibit circadian rhythmicity, though the most easily observed is a simple measure of when an organism is active or inactive. The complexity of circadian rhythm goes far beyond this, however, and it is this complexity which makes time of day a key factor in pharmacology, one that requires quantifying. As mentioned above [section 3.2], levels of receptor expression are one pharmacologically relevant parameter that can vary in circadian manner, in this case the expression of α_1 and α_2 adrenergic receptors in the PVN (Jhanwar-Uniyal *et al.*, 1986; Morien *et al.*, 1999). Clearly such variation is of direct relevance to pharmacological study, as variation in receptor populations will mean variation in the response to agents which act via these receptors. This is just one example of how circadian rhythms may affect drug treatment and thus a brief discussion of circadian rhythmicity and why investigating these effects is important follows.

The central control of the circadian system is accepted as being located in the SCN, within the hypothalamus; this is demonstrated by the fact that in animals where the SCN is ablated all behavioural and physiological rhythms are lost if photic cues – changes in light – are removed (Weinert, 2005; Weaver, 1998). One of the neurotransmitters heavily implicated in the circadian system is 5-HT, and this transmitter and various receptors for it are prevalent in the SCN (Rae, 1998). Activation of certain subtypes of 5-HT receptors (*e.g.* 5-HT_{1A}, 5-HT_7; Biello and Dafters, 2001; Lovenberg *et al.*, 1993; Rae, 1998) can cause "phase shifts" – temporal shifts of activity rhythms observed in constant light or darkness – in animals isolated from zeitgeibers (the environmental cues that entrain the internal clock). Interestingly direct injection of 5-HT into the SCN also alters feeding behaviour (mentioned in their review of serotonin and feeding behaviour by Leibowitz and Alexander, 1998). For more complete understanding of the SCN, the reader is referred to the special issue of *Chronobiology International* (Volume **15**, Issue 5, 1998) devoted to reviews concerned with this nucleus, and to other recent reviews (Weinert, 2005; Weaver, 1998).

Of more direct relevance to this thesis is how circadian rhythm can influence pharmacology; specifically how such variation may interact with interventions in central nervous system control of feeding and satiety. One such mechanism – variation in receptor expression – has been mentioned above; changes in the balance of α_1 and α_2 adrenergic receptors could have a significant impact on the efficacy of sibutramine [section 3.4], which blocks NA (and 5-HT) re-uptake. Adrenergic receptors are not the only receptor type that exhibit circadian rhythmicity, nor are changes in the level of receptor expression the only mechanism by which rhythmicity may be expressed. For example, Garabette *et al.* (2000) found circadian variation in the functional activity of SCN 5-HT_{1B} receptors, although no variation in levels of expression had been reported.

Perhaps yet more relevant is the natural circadian variation in levels of the neurotransmitters themselves – whether of tissue levels, extracellular levels, or the rate of synthesis or release. Both NA and 5-HT levels vary over 24 hours, although 5-HT more markedly than NA (see the review by Martin and Redfern, 1997). Both transmitters are recorded as having pronounced circadian variation in tissue levels, but

the more important differences appear to be in the nature of the variation in release. The circadian variation in release of NA is very closely tied to overall arousal and behavioural activity, at least in the hippocampus (Kalen *et al.*, 1989); consequently in nocturnal animals like rats the basal level of NA was high during the dark phase and lower during the light. Telencephalon NA levels – intra and extracellular – match this pattern (Philo *et al.*, 1977). In addition to this, NA levels in the PVN showed short-lasting peaks at the beginning and end of the dark phase; these peaks disappeared in food deprived animals, replaced with a slow accumulation of NA until the animals were fed, at which point the levels of NA dropped immediately (Stanley *et al.*, 1989). This suggests that PVN levels of NA are driven by external factors and the rhythmicity of feeding behaviour as opposed to being endogenously generated on a circadian basis.

By contrast, tissue levels of 5-HT are highest during the light phase in the rat; however this does not translate to a pattern of release or usage. Further investigation revealed that 5-HT synthesis was greatest in the light, but breakdown greatest during the dark phase. This finding by Hery *et al.* (1972) was the first to suggest that more 5-HT was used and released during the active phase, and was backed up by *in* vivo voltammetry studies by Faradji *et al.* (1983). Also in contrast to NA there are indications that rhythms in 5-HT levels are endogenously generated (Cagampang and Inouye, 1994), at least in the SCN. As is seen with locomotor activity rhythms (*e.g.* Davies *et al.*, 1972), rhythms in 5-HT levels – at least in the SCN – "free run" when animals are moved into constant darkness (Cagampang and Inouye, 1994). Further study showed that there are circadian variations in almost every function of 5-HT turnover – from synthesis, to neuronal firing rate, to receptor function and ultimately metabolism (see Martin and Redfern 1997 for a complete review). Given the primary neurotransmitter(s) on which both fenfluramine and sibutramine act exhibit such a wide array of circadian changes it is not a large conceptual leap to expect circadian variation in the response to these drugs. Additionally whilst most is known about how 5-HT levels vary with circadian time, and NA levels are also known to vary, it is reasonable to expect that a similar degree of variation may be seen with other neurotransmitters, including others with an influence – however tangential – on feeding behaviour, such as dopamine or some of the peptide neurotransmitters.

Variation in transmitter levels is not the only way in which the efficacy of these drugs may be affected by the time of day at which they are administered. Circadian variation in pharmacokinetic properties has been reported with many drugs, from common aspirin, to penicillins, to cardiovascular therapies, to centrally acting compounds like diazepam (Labreque and Bélanger, 1991; Reinberg and Smolensky, 1982). This is perhaps especially relevant to the current work, as sibutramine is a parent compound with not just one, but two active metabolites. It is these metabolites which are responsible for the majority of the *in vivo* actions of sibutramine to inhibit reuptake of NA and 5-HT [section 3.4]. Fenfluramine also has a metabolite that contributes to its therapeutic use; whilst fenfluramine itself functions as a re-uptake blocker at low concentrations and 5-HT releasing agent at higher concentrations [section 3.5], its metabolite norfenfluramine is a direct agonist at 5-HT_{2C} receptors (Gibson *et al.*, 1993; Simansky, 1996). Circadian rhythms in the breakdown of the parent compounds could thus potentially contribute significantly to circadian variation in the efficacy of treatment. Labrecque and Bélanger (1991) reviewed why circadian rhythm in pharmacokinetics is an important factor to consider in pharmacology.

Finally, another obvious reason why time of day might be a significant factor in the efficacy of anti-obesity therapy, and specifically therapies that act by enhancing natural satiety, is that standard activity rhythms – including those associated with feeding – are under fairly stringent circadian control. Rats consume the vast majority of their daily intake in the dark phase when they are also most behaviourally active. It would thus appear intuitive to target treatment towards times of peak intake.

With the above thoughts in mind it is now appropriate to consider the drugs to be investigated in this thesis.

3.4 Sibutramine

Sibutramine hydrochloride (1-(4-chlorophenyl)-N,N-dimethyl-a-(2-methylpropyl)-cyclobutanemethanamine) is a 5-HT and NA reuptake inhibitor. Developed by Boots Pharmaceuticals (Nottingham, UK), originally with a view to producing a rapidly acting anti-depressant, sibutramine has been investigated and used as a novel anorectic (anti-obesity) agent (Heal *et al.*, 1998*a*). The following is a brief overview of this drug, its pharmacology, experimental history and (pre-)clinical effects.

Sibutramine (**figure 3.6.3**) exerts its effects primarily through two active metabolites – primary and secondary amines (desmethyl- and di-desmethyl- sibutramine) – which are also both active as inhibitors of NA and 5-HT reuptake and more potent than the parent compound (**table 3.6.1**; Heal *et al.*, 1998*a*; Luscombe *et al.*, 1989). Upon administration sibutramine is rapidly demethylated in the liver and it is subject to extensive first pass metabolism if given orally whilst the metabolites have long half-lives; in man the pharmacokinetics are not variable with age (*e.g.* Hind *et al.*, 1999). In the rat similar extensive first-pass metabolism occurs if the drug is administered either orally or *i.p.*, so much so that by 30 minutes after administration sibutramine plasma concentrations are very low, whilst the concentrations of both metabolites are at close to peak levels (personal communication, D. Heal). Buckett *et al.* (1988) describe experiments where sibutramine was examined by *in vivo*, *ex vivo* and *in vitro* experimental models designed to give indication of therapeutic potential for the treatment of depression. These authors found that sibutramine was potently active in behavioural paradigms predictive of anti-depressant activity, as well as being capable of rapid down-regulation of β-adrenoreceptors – a functional change believed very important in depressive therapy at the time (Vetulani and Sulser, 1975). The compound was further developed and made it to phase II clinical trials where, against expectation, it failed to exhibit anti-depressant efficacy. However, a side effect of treatment with sibutramine in these trials was that patients lost weight (*e.g.* Heal *et al.*, 1998*a*); this finding has since been replicated in many other clinical trials (Weintraub *et al.*, 1991) and the drug has since been widely studied with respect to anti-obesity therapy (as reviewed by Heal *et al.*, 1998*a*).

The results of numerous experiments illustrate that sibutramine and its metabolites work solely via re-uptake inhibition. Rather than stimulating release of NA or 5-HT from neurons – via electrical stimulation, by transporter reversal or other mechanism – sibutramine leads to a slow build up of extracellular neurotransmitter (Gundlah *et al.*, 1997; Heal *et al.*, 1998*a,b*) as the transmitter released by nerve firing simply cannot be removed from the extracellular fluid. This is in contrast to fenfluramine which causes the release of stored 5-HT (Gundlah *et al.*, 1997; Heal *et al.*, 1998*a,b*; section 3.5). Comparatively the magnitude of change in extracellular levels is much greater with releasing agents than it is with re-uptake inhibitors and the maximal extracellular concentration is also reached sooner (Gundlah *et al.*, 1997; Heal *et al.*, 1998*a,b*). The ability of a re-uptake inhibitor to elevate extracellular 5-HT is therefore potentially more likely to exhibit circadian variation in efficacy than might be the case for a releasing agent. Re-uptake inhibition requires endogenously-generated neuronal firing to increase extracellular 5-HT, whilst suppressing such firing by ensuring the continued presence of 5-HT in the synapse, where it can activate terminal autoreceptors (Gundlah *et al.*, 1997; Heal *et al.*, 1998*a,b*), thus exerting a negative feedback on neuronal 5-HT release.

Sibutramine induces weight loss by working on both sides of the energy balance. Not only does it reduce food intake by enhancing satiety (Halford *et al.*, 1995; Halford *et al.*, 1998; Heal *et al.*, 1998*a*) but it also induces an increase in energy usage by promoting thermogenesis in brown adipose tissue, raising basal metabolic rate (Connoley *et al.*, 1999; Heal *et al.*, 1998*a*; Stock, 1997). Both facets of this action involve synergistic interactions of NA and 5-HT and are sensitive to a variety of antagonists of adrenergic and serotonergic systems (see below). The work presented in this thesis will concentrate on the satiety enhancing actions of sibutramine. The reader is advised to look to other reviews and publications for further information on the thermogenic properties of sibutramine (*e.g.* Connoley *et al.*, 1999; Heal *et al.*, 1998*a*; Stock, 1997).

Before going on to consider the satiety enhancement it is pertinent to describe more fully the pharmacology of sibutramine and its metabolites. **Table 3.6.1** (reproduced from data reported in Heal *et al.*, 1998*a*) indicates the potencies of sibutramine and both of its metabolites – as well as those of related compounds and the anorectic

monoamine releasing agents *d*-amphetamine and *d*-fenfluramine [section 3.5 below] – to inhibit re-uptake of monoamines *in vitro* in tissue collected from rat brain. Although this shows the relative selectivity for 5-HT and NA over dopamine, the data presented suggest that dopamine re-uptake might be a contributing factor to the pharmacology of sibutramine. However, Heal *et al.* (1992) used a drug discrimination paradigm to show that sibutramine generalised to saline rather than amphetamine; these authors additionally showed that sibutramine did not mimic dopaminergic agents in inducing circling behaviour in rats with unilateral lesions of the nigrostriatal pathway. There have been findings that sibutramine did indeed increase extracellular dopamine in the striatum and hypothalamus of rats (Balcioglu and Wurtman, 2000; Rowley et al., 2000), but these effects were observed with relatively high doses of sibutramine. Administration of sibutramine at doses close to the ED_{50} for inhibition of 2 hour food intake (2.0 mg/kg; Halford *et al.*, 1995; Rowley *et al.*, 2000) caused no significant increase of extracellular dopamine (Balcioglu and Wurtman, 2000; Rowley *et al.*, 2000). Even at doses at which sibutramine did induce a significant increase in extracellular dopamine there was no evidence of an effect on locomotor activity (Rowley *et al.*, 2000). Further evidence of a relative lack of dopaminergic contribution to the actions of sibutramine is that the compound appears to lack the abuse potential of amphetamine (Gosden *et al.*, 1996; Heal *et al.*, 1998*a*; Schuh *et al.*, 2000).

Sibutramine is accepted as producing its inhibition of food intake by advancing the behavioural satiety sequence (Halford *et al.*, 1995; Halford *et al.*, 1998; Heal *et al.*, 1998*a*) – indicative of the drug enhancing the natural process of satiation. Jackson *et al.* (1997*a*) investigated the contribution of various receptor subtypes to 8 hour sibutramine hypophagia and found roles for α_1 but not α_2, and for β_1 but not β_2, adrenoreceptors, as well as for $5\text{-HT}_{2A/2C}$ receptors. As described above [section 3.1], activation of α_1 receptors in the PVN suppresses feeding (Leibowitz and Hoebel, 1998; Wellman *et al.*, 1993). Given this, it is unsurprising that blocking this receptor negated the hypophagic effect of a drug that induces a rise in extracellular NA levels (Jackson *et al.* 1997*a*), especially given that endogenous NA preferentially activates α_2 receptors over α_1 receptors (Wellman *et al.*, 1993) and α_2 receptor activation in the PVN can stimulate feeding even in satiated rats (Goldman *et al.*, 1985). It is also unsurprising that a partial attenuation of sibutramine hypophagia can be achieved by

blockade of 5-HT_2 receptors – and the 5-HT_{2C} subtype in particular; the activation of these receptors has been shown to reduce the rate of feeding in rats (Clifton, 2000; Grignaschi and Samanin, 1992; Simansky, 1996; section 3.2). This work with selective antagonists complements more work by Jackson *et al.* (1997*b*) using the 5-HT uptake inhibitor fluoxetine in combination with the NA uptake inhibitor nisoxetine. These experiments showed that while re-uptake inhibition of either NA or 5-HT alone was not sufficient to reproduce the effects of sibutramine, in combination these drugs were able to inhibit food intake in a manner consistent with the effect of sibutramine, and with similar potency.

3.5 Fenfluramine

In addition to sibutramine [described in section 3.4 above] the other drug central to the experiments described in this thesis is the *d*-isomer of fenfluramine (*N*-ethyl-1-[3-(trifluoromethyl)-phenyl]propan-2-amine).

Fenfluramine bears some chemical relation to amphetamine (**figure 3.6.4**), whilst exhibiting a very different pharmacological profile – despite both having a proven record as anorectic agents (Ziance *et al.*, 1972; Dobrzanski and Doggett, 1976; Thurlby *et al.*, 1983; Rowland and Carlton, 1986; Heal *et al.*, 1998*a*). Where amphetamine is primarily active upon the catecholamines, NA and dopamine, fenfluramine is primarily a serotonergic agent though with some lesser impact on catecholaminergic systems (Ziance *et al.*, 1972; Rowland and Carlton, 1986; Heal *et al.*, 1998*a*). In terms of its affinity for, and efficacy at inhibiting, the uptake carrier *d*-fenfluramine is approximately equipotent at the NA and 5-HT carriers (*e.g.* **table 3.6.1;** Heal *et al.*, 1998*a*), however there is a significant separation in the concentrations at which *d*-fenfluramine is active at releasing serotonin and noradrenaline, Whilst there is only a 3-fold difference in the concentration required to block re-uptake or stimulate release of 5-HT, there is a 40-fold separation between the concentration of fenfluramine required to block NA re-uptake and that required to cause significant NA release, thus in terms of its potential to cause monoamine release fenfluramine is selective for serotonin (Heal *et al.*, 1998*a*).

Amphetamine increases spontaneous locomotor activity, whilst fenfluramine decreases the same parameter (*e.g.* Ziance *et al.*, 1972; section 5.1). Similarly, where amphetamine is associated with high potential for abuse, fenfluramine is not (Rowland and Carlton, 1986) and where amphetamine disrupts the behavioural satiety sequence fenfluramine enhances it (*e.g.* Halford *et al.*, 1998) – although there have been contradictory reports upon fenfluramine's action in the past [see section 4.1]. Interestingly, too, racemic fenfluramine actually has a negative impact on dopamine systems, thought to be a result of receptor antagonism mediated by the *l*-isomer (Rowland and Carlton, 1986). This is just one contributory reason why the *d*-isomer has replaced the racemate as the primary form in which fenfluramine is used.

Yet despite these differences there are also important similarities between amphetamine and fenfluramine. Both are monoamine releasing agents. Like amphetamine, fenfluramine binds to re-uptake sites (see **table 3.6.1**) – indeed fenfluramine acts as a 5-HT re-uptake inhibitor at low concentrations (Rowland and Carlton, 1986; Garattini *et al.*, 1986). Both compounds enter neurons, where they stimulate release from intracellular stores - an effect seen *in vivo* and replicated in brain slices *in vitro* (Garattini *et al.*, 1986; Sulzer *et al.*, 2005; Wortley *et al.*, 1999; Heal *et al.*, 1998*a,b*; Gundlah *et al.*, 1997). Therefore fenfluramine causes an increase in extracellular 5-HT levels independently of neuronal firing, as amphetamine does with both dopamine and NA; fenfluramine also releases NA but with much lower potency (see **table 3.6.1** and above; Rowland and Carlton, 1986). In fact, binding to the re-uptake transporter is required for fenfluramine entry into neurons as pre-treatment with a selective inhibitor of these sites (like fluoxetine or indeed sibutramine) attenuates fenfluramine-induced 5-HT release (Gundlah *et al.*, 1997). The *d*-isomer has greater affinity for the carrier than the *l*-isomer (Rowland and Carlton, 1986).

Fenfluramine - and so too, separately, the *d*-isomer - had previously been licensed for the clinical treatment of obesity in the USA but both were voluntarily withdrawn in 1997 following concerns over cardiovascular side-effects. In rodents the drug works primarily by reducing food intake, mediated by a 5-HT-dependent enhancement of post-ingestive satiety – an effect determined by several experiments observing the behavioural satiety sequence [section 4.1]. The *d*-isomer is more potent in terms of reduction of food intake (Rowland and Carlton, 1986), which is the primary clinical effect of taking fenfluramine, and the *d*-isomer of norfenfluramine – a fenfluramine metabolite – is similarly more potent than its *l*-counterpart. In fact, *d*-norfenfluramine is a direct agonist at 5-HT_{2C} receptors (Gibson *et al.*, 1993) which, together with its long half-life (*e.g.* Rowland and Carlton, 1986; Garattini *et al.*, 1986), contributes significantly to the anorectic potential of *d*-fenfluramine as this receptor type is implicated in feeding behaviour, specifically with reference to the rate of eating (Grignaschi and Samanin, 1992). That *d*-fenfluramine itself may have some direct agonist potential on some yet unconfirmed receptor is also a possibility given that the hyperphagia produced by *d*-fenfluramine is not blocked by pre-treatment with 5-HT

reuptake inhibitors but is by the non-selective 5-HT antagonist metergoline (Raiteri *et al.*, 1995; Curzon *et al.*, 1997).

Another factor that may contribute to the efficacy of fenfluramine in treating obesity is that it has transient effects on muscular energy expenditure. Even and Nicolaidis (1986) discovered that *d*-fenfluramine increased the energy cost of muscular effort. Not entirely unrelated to this are reports of fenfluramine as a sedative, as measured by recordings of locomotor activity (Ziance *et al.*, 1972; Aulakh *et al.*, 1988; Callaway *et al.*, 1993), though significant sedation is not thought to play a role in the reduction of food intake by fenfluramine, and the drug does not sedate heavily enough at appropriate anorectic doses to disrupt the behavioural satiety sequence [chapter 4]. Moreover, the sedative or motor-depressant actions of fenfluramine have been shown to be mediated by a different pathway to the effects on food intake (Callaway *et al.*, 1993). On top of all this, fenfluramine has been shown to increase diet-induced thermogenesis, although this effect has only been reported with doses well above those required for hypophagia (Rowland and Carlton, 1986) and it is not thought that this is physiologically relevant to the anorectic effects of fenfluramine. Another interesting effect of fenfluramine is that if it is given to animals *after* they have eaten to satiation they experience a taste aversion reaction and eat less the next day (Rowland and Carlton, 1986).

The four pharmacological criteria that Gundlah *et al.* (1997) defined for differentiating 5-HT re-uptake inhibitors and releasing agents *in vivo* proved sibutramine and its primary amine metabolite were acting as reuptake inhibitors. The same criteria also provide evidence that fenfluramine acts primarily as a releasing agent *in vivo*.

These criteria (Gundlah *et al.*, 1997) are:

- The magnitude of the change in extracellular neurotransmitter levels; the increase is much higher with releasing agents.
- The dependence on neuronal activity; re-uptake inhibitors rely on neuronal activity to elevate extracellular transmitter level

- Whether pre-treatment attenuates the effect of known releasing agents dependent on the 5-HT carrier; re-uptake inhibitors prevent entry of these releasing agents into the neuron, thus reducing the magnitude of their effect, whilst releasing agents do not.
- Whether the systemic administration of a drug during an intra-hypothalamic infusion of that same drug attenuates the large initial increase seen on local infusion; peripheral administration of a re-uptake inhibitor during its local infusion will reduce the magnitude of the change in extracellular transmitter levels

The recorded rise in extracellular 5-HT is too fast and of too great a magnitude for it to be due to re-uptake inhibition (Gundlah *et al.*, 1997; Heal *et al.*, 1998*b*). A degree of re-uptake inhibition may well be involved in limiting the rate at which the released 5-HT is cleared, however. The levels of release obtained with fenfluramine are so high compared to basal levels of 5-HT (*e.g.* Gundlah *et al.*, 1997; Heal *et al.*, 1998*b*) that one might expect less circadian variation in the response to be caused by changes in 5-HT availability than might be attributable to postsynaptic receptor changes. That the *in vivo* hypophagic response to fenfluramine is not dependent on 5-HT availability (though 5-HT release could still play a role in normal mediation of the response; Curzon *et al.*, 1997) would tend to suggest that any circadian variation in the response to fenfluramine would be more likely to be dependent on 5-HT receptor variations than on tissue levels of 5-HT (highest in the light phase), the rate of 5-HT synthesis or on the basal activity of serotonergic circuits. On the other hand, since fenfluramine is reported as having additional actions other than to enhance satiety – namely the sedative and metabolic effects described above – there is every chance that these parameters may exhibit some level of circadian variation especially since the effects on motor activity can be functionally dissected from the hypophagic effect (Callaway et al., 1993). This means that there are at least two distinct mechanisms in which circadian variation may influence drug action, on top of any circadian changes in pharmacokinetics. There are clinical reports of lower doses of fenfluramine (in the common "fen-phen" combination with phentermine) proving effective at inducing weight loss when given in the afternoon (Katz *et al.*, 1999) and as discussed above [section 2.3] circadian variation in efficacy may be tied more to rhythms in natural

feeding behaviour and activity than directly to effects on neurotransmitters. Fenfluramine has also shown to inhibit food intake differentially at various points in the circadian cycle (Davies and Wellman, 1991),

3.6 Figures for Chapter 3

Figure 3.6.1 The chemical structure of noradrenaline

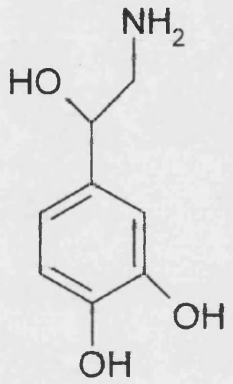


Figure 3.6.2 The chemical structure of 5-hydroxytryptamine (5-HT)

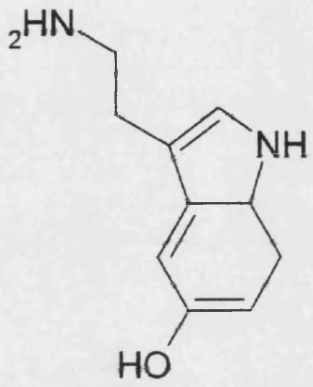


Figure 3.6.3 The chemical structure of sibutramine and its two active metabolites

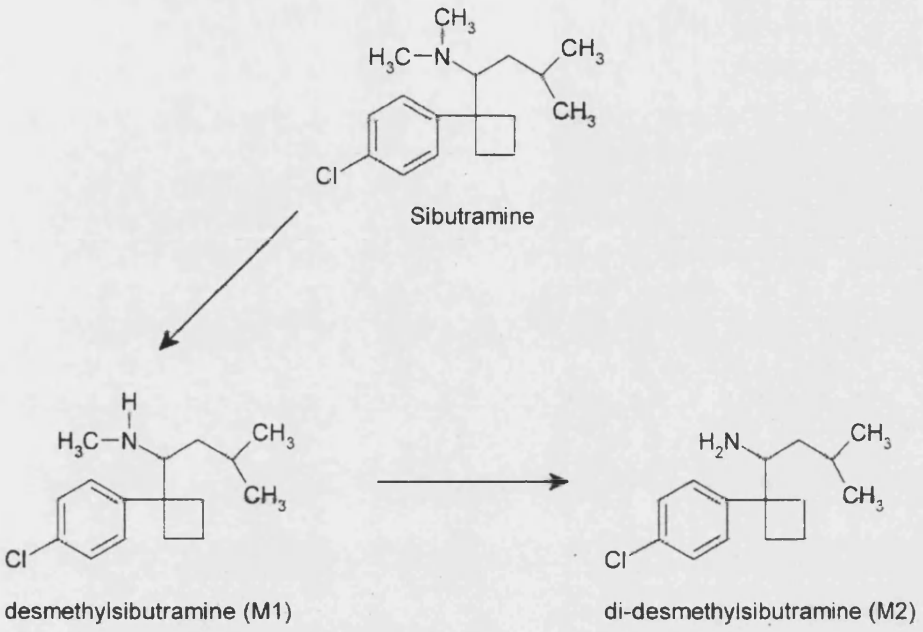
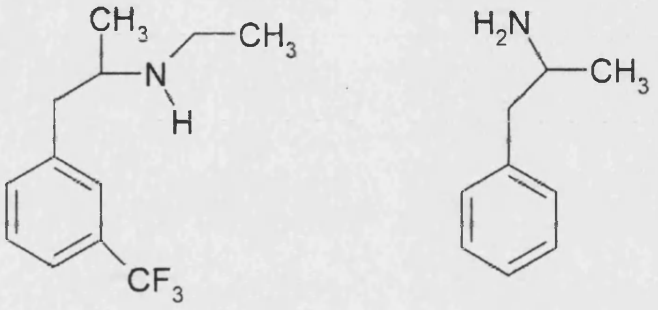


Figure 3.6.4 The chemical structures of fenfluramine (left) and amphetamine



Fenfluramine Amphetamine

Table 3.6.1 *In vitro* inhibition constants for the re-uptake of monoamines by sibutramine and its metabolites compared to other monoamine re-uptake inhibitors and other anti-obesity drugs.

Compound	Noradrenaline	Serotonin	Dopamine
Sibutramine	283	3131	2309
Metabolite 1	2.7	18	24
Metabolite 2	4.9	26	31
Nisoxetine	2.1	296	279
Fluoxetine	320	11	2025
d-Amphetamine	45	1441	132
d-Fenfluramine	260	279	6227

Values are the nanomolar (nM) concentrations for the inhibition constant (Ki). Metabolite 1 is the secondary amine (desmethylsibutramine) and metabolite 2 is the primary amine (didesmethylsibutramine; **figure 3.6.3**). This table is reproduced from data found in Heal *et al.* (1998*a*).

4.0 Calibration of the Behavioural Satiety Sequence (BSS) for Circadian Work

4.1 Introduction

4.1.1 What is the BSS and how does it help discriminate the mechanisms of drug action?

The behavioural satiety sequence is a well characterised method for investigating the anorectic activity of drugs. Initially described by Antin *et al.* (1975), the BSS is a preserved behavioural pattern exhibited as feeding animals satiate. It is important to note, however, that the interpretation of the BSS is based on trends and behavioural patterns over time rather than a rigid, temporally-determined progression which never varies. The sequence, a progression from initial feeding via brief periods of activity and grooming to a state of post-prandial resting, is specific to satiety and satiation rather than feeding *per se.* It is not seen with sham feeding, wherein ingested food is drained from the stomach via an implanted cannula, although sham feeding rats will exhibit the full satiety sequence if given cholecystokinin (CCK). This finding, by Antin *et al.* (1975), when coupled to their observations of a behavioural progression in normal animals, was responsible for highlighting the role of CCK in natural satiety. Their findings were also a fundamental on which a lot of subsequent research into satiety has been built.

Since the BSS was first described it has been used extensively for examining the impact of drugs on feeding behaviour. As the BSS is a naturally occurring phenomenon the manner in which pharmacotherapies alter the expression of the behaviours involved can inform on their physiological effects and the mechanisms underlying them. In the laboratory, where the conditions associated with testing, observation and food intake can be kept constant, it is reasonable to suggest that any changes seen in the profile of the BSS are the result of factors consciously changed by the researchers – such as drug treatment. Behavioural changes help elucidate pharmacology in many fields of drug research –especially with regards to the central nervous system, *e.g.* research into addiction, depression, neurodegeneration etc. (*e.g.* Shoaib and Stolerman 1999; Mitchell 2005; Mitchell and Redfern 2005; Yamada and Nabeshima 2000 – and the BSS is no exception. Drugs with different mechanisms of

action can produce different changes to the profile of the BSS, and the nature of these changes gives an insight into the physiological properties of the drug. Appetite suppression can be achieved through a number of different mechanisms such as making food unpalatable, sedation, hyperactivity or the enhancement of natural satiety. These distinct physiological mechanisms manifest differently in the BSS, each producing characteristic changes. Those effects that alter the behavioural profile such that the characteristic sequence is not seen are said to disrupt the BSS.

Amphetamine has long been used as pharmacotherapy against over-eating, as have analogues such as phentermine (Kordik and Reitz, 1999); the major contributing mechanism of action for amphetamine is to cause release of stored noradrenaline and dopamine from neurons (Heal *et al.*, 1998*a*; Sulzer *et al.*, 2005; Wortley *et al.*, 1999). One of the primary consequences of this is a general increase in activity levels, and it is this hyperactivity that is chiefly responsible for the reduction in food intake in rats dosed with amphetamine (Halford *et al.*, 1995, 1998; Halford, 2001). Such hyperactivity would also have beneficial effects for weight loss through other means, as more energy is expended during the hyperactivity as well as less food consumed. When looked at in the BSS, this reduction results from a displacement of feeding behaviour to other forms of active behaviour; similarly such hyperactivity is not compatible with long periods of resting behaviour, and the onset of resting is delayed (Halford *et al.*, 1998). Moderate increases in general activity levels may not necessarily disrupt the BSS. Significant hyperactivity on the other hand, whether displacing feeding behaviour as with amphetamine or delaying resting behaviour as with the intra-accumbens administration of moderate doses of the direct dopaminergic agonists SKF-38393 (D_1 receptors) and LY-171555 (D_2 receptors) (Philips *et al.*, 1995) or systemic administration of the non-subtype-selective 5-HT_2 receptor agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI; Kitchener and Dourish, 1994), is easily determined by observation and causes a disruption of the BSS.

Significant sedation, such as that seen with high doses of the 5-HT_2 receptor agonist MK-212, manifests in the BSS by a displacement of feeding and active behaviours by resting behaviour (Halford *et al.*, 1996*a*). As with moderate increases in activity, moderate sedation may result in a seemingly preserved satiety sequence but a reduced

expression of active behaviours, such as that seen with fluoxetine by Halford and Blundell (1996*b*).

Addition of quinine (or other unpalatable compounds) makes food distasteful to rats (Antin *et al.*, 1975), altering the BSS profile; satiation is delayed, if indeed it is reached at all. This is because the animals make a large number of short, un-sustained trips to the food source, leaving it aside due to its unwelcome taste and exhibiting other active behaviours instead, thus not consuming enough food to satiate in the same way that animals presented with untreated food would.

Satiety enhancing drugs are compounds that facilitate the natural process of satiation and satiety. Drugs that act in this manner produce a temporal shift in the BSS, with both the offset of feeding and the onset of resting occurring earlier in the observation period (Halford *et al.*, 1998). This satiety enhancement matches the change in the profile of the BSS produced by pre-feeding (Halford *et al.*, 1998). Pre-feeding implies the return of food a while before the observations begin, thus animals are part-fed as the observation commences and naturally satiate sooner. This kind of change is referred to as an advancement of the BSS. Several drugs fall into this category, including the selective serotonin re-uptake inhibitors (SSRIs) fluoxetine (Clifton *et al.*, 1989; Halford and Blundell, 1996b; Halford *et al.*, 1998; McGuirk *et al.*, 1992*a*; Willner *et al.*, 1990), paroxetine and femoxetine (McGuirk *et al.*, 1992*b*), the serotonin and noradrenaline re-uptake inhibitor (SNRI) sibutramine (Halford *et al.*, 1995, 1998) and with the serotonin releasing agent (*d*-)fenfluramine (Halford *et al.*, 1995, 1998). This last drug was, for a while, a subject of debate as to whether it advanced or disrupted the BSS (McGuirk *et al.*, 1992a; Montgomery and Willner, 1988; Willner *et al.*, 1990), but the balance of evidence and the current thinking is for a satiety-enhancing (and BSS-advancing) action for (*d*-)fenfluramine.

This list of compounds implicates 5-HT as quite heavily involved in natural processes of satiety [section 3.1], and many other drugs which act primarily through 5-HT systems also preserve and advance the BSS, including other re-uptake inhibitors and direct agonists at receptor subtypes (see Halford *et al.*, 1998, for a review of drug action and the BSS). Not all drugs acting on serotonergic systems advance and preserve the BSS however; as noted above MK-212 and DOI are two examples of

serotonergic drugs which disrupt the BSS. Nor are serotonin enhancing drugs the only type of compound capable of advancing and preserving the BSS, with antagonists of the peptide neurotransmitter orexin-A (Rodgers *et al.*, 2001) now known to do likewise; orexin-A, in contrast, delays the BSS (Rogers *et al.*, 2000); the peripheral satiety signal molecule CCK (Antin *et al.*, 1975) and others compounds, including peripherally administered 5-HT (Edwards and Stevens, 1991; Simansky, 1996), have also been shown to advance and preserve the BSS.

4.1.2 How to measure the BSS

How to observe, record, and interpret the BSS has been a matter of debate. There are two primary schools of thought, one espoused by Halford, Blundell and colleagues (Halford *et al.*, 1995, 1996, 1998; Halford and Blundell 1996*a*,*b*; Rodgers *et al.*, 2000, 2001) of continuous analysis from videotaped observation sessions and the alternative of some form of time-sampled observations as utilised initially by Antin *et al.* (1975) and adapted since then by many groups (*e.g.* Clifton *et al.*, 1989; Montgomery and Willner, 1988; McGuirk *et al.*, 1992*a*,*b*; Lee and Simansky, 1997). In their review of the BSS and its contribution to the behavioural pharmacology of anorectic drugs, Halford *et al.* (1998) perhaps unsurprisingly backed their continuous analysis methodology as superior, and all things being ideal it does offer advantages as laid out in that review; continuous analysis provides a complete behavioural picture with full details of the duration and frequency of each behaviour recorded, which no time-sampling method can match. However, while the more in-depth nature of the continuous analysis makes it a much more accurate, it also makes carrying out BSS experiments a lot more demanding, and increases the reliance on specialised apparatus. Compared to a time-sampling method it reduces the number of animals used but therefore leaves the results open to larger distortions caused by non- or poorly-responding animals – including those which, for any reason, do not feed whilst under observation. Coupled to the more time-intensive nature of the analysis and the greater reliance on a specialised set-up this makes continuous analysis a technique best suited to dedicated laboratories doing a large body of such work.

None of which is to say that time sampling does not have its own faults. The behavioural picture achieved with time-sampling is far from complete and most such methods track far fewer behavioural categories than would be possible with continuous analysis. There is also the question of observer bias, where transitions of behaviour are often scored in favour of frequent but short-lasting behaviours (*e.g.* locomotion) over longer lasting, less frequent behaviours such as resting; this is known as "event over state observer bias" (Halford *et al.*, 1998). On the other hand, time-sampling does facilitate the testing of many animals at once and findings from studies using time-sampling have provided valuable information on a number of compounds (*e.g.* fluoxetine; Clifton *et al.*, 1989) that has later been corroborated by

continuous analysis (Halford and Blundell, 1996*b*; Halford *et al.*, 1998). It is therefore important to determine what one is looking for before embarking on a prolonged sequence of BSS experiments, and evaluate which method is more appropriate to the task at hand. For studies where an in-depth record of total behaviour or behavioural microstructure is required then it is clear that continuous analysis is the way to go. Whether such an intensive technique is necessary to identify trends and changes in behavioural macrostructure is another matter, especially if there are issues in assembling a unit capable of supporting such detail.

Thus the first steps taken in this study were to evaluate whether a time-sampling technique with behavioural coding done during the observation period, or a more detailed approach using analysis from videotaped sessions, would be appropriate for investigating the BSS in relation to sibutramine, *d*-fenfluramine and circadian rhythm [chapter 7 (below) describes this circadian work], and once decided, to find appropriate doses of the drugs to use in future work and to establish a common protocol for the experiments to come.

4.2 Materials and Methods

All experiments described in this thesis were carried out in accordance with The Animals Scientific Procedures Act 1986.

The following were maintained across the BSS experiments to be described in this thesis (here and chapter 7) except where specifically countered.

Animals. Male Wistar rats from the University of Bath breeding colony (150-280 grams at the start of experiments) were used in all procedures described here. Animals were housed in pairs with *ad libitum* access to food (CRM(E) pellets; Special Diets Services, Witham, UK) and water. The room was maintained at 21°C (+/- 1°C) and on a regular 12:12 hour light/dark cycle, with a 15 Watt red light bulb mounted above each cage providing illumination during the dark phase.

Drugs. Sibutramine hydrochloride monohydrate and *d*-fenfluramine hydrochloride (RBI, donated by RenaSci (Nottingham, UK)) were both dissolved in 0.9% saline (Fresenius Kabi, Warrington, UK). Drug solutions were made up freshly each day and all injections were made with 2 ml/kg of the drug solution or vehicle into the peritoneum, such that doses outlined in the individual experiments below were administered.

General Procedure. 12 animals were weaned and separated into paired housing as described above where they were left to habituate to the new surroundings for 2 weeks. On the third week the process of habituating animals to the experimental chambers and procedures began. Animals were isolated into roofless cages (dimensions 40 x 56.5 cm, with solid sides and a clear Perspex front 89.5 cm high, with the bottom tray sliding forward to allow access; **figures 4.5.1** to **4.5.3**) 24 hours before observations began, still with free access to food and water. Six hours prior to observation, animals were deprived of food by sliding a metal plate down to block access to the food hopper; animals retained free access to water at all times. Animals would also be weighed at this time and returned to the observational cage. Thirty minutes prior to observation animals would be dosed with drug or saline as appropriate, and once more returned to the observational cage. When the time of

observation arrived food, in the form of ground up standard chow presented in feeding jars (10.5 cm diameter, 9 cm diameter at rim; **figure 4.5.4**) with a 4 cm diameter hole cut into the lid through which the food was accessible, was re-introduced to the cage and recording began. For the next 60 minutes each animal was observed sequentially for 5 seconds in every 30, and the predominant behaviour exhibited in that time was scored by hand into one of four mutually exclusive categories, similar to those used by Clifton *et al.* (1989). These were: feeding (eating, drinking, showing active interest in food or water source), grooming (licking, scratching, washing), resting (non-moving or still, awake or asleep) or active (activities such as sniffing, rearing and locomotion not covered by the above). This active category was reserved for active behaviours not covered explicitly by feeding or grooming. Six animals were observed at any one time, and a total of 120 observations were made for each animal. After 60 minutes animals were returned to their home pair with free access to food and water. The food jars were weighed before and after presentation to the animals and the difference in the readings was recorded and assumed to represent food intake.

Each animal was tested once per week, with 6 animals observed per testing session. Animals were used as their own controls, and were tested in random treatment order. Experiments lasted until each animal had been given each treatment once.

Prior to any scored runs, animals were given 3 weeks of habituation to the procedure. Feeding from the food jars was a learnt behaviour, as the form the food was presented in differed from that the animals received in the home cage. Therefore the first 2 exposures to the observation cages were accompanied by a 24 hour food deprivation in order to further stimulate interest in the presented food jars and aid the learning process. On these occasions the animals were not observed. In the final week of habituation the deprivation was reduced to six hours, and all animals were dosed with saline (2 ml/kg *i.p.*); animals were actively observed (though not scored) following presentation of the food jars on this occasion.

Statistical analysis. For analysis, the scored behaviours were transferred into a spreadsheet and tallied into 5-minute epochs. One way ANOVAs with *post-hoc* Dunnett's tests were used on cumulative data from 5 minute epochs through to the full 60 minute period; these examined each behaviour independently, with feeding and

resting behaviour of primary interest. Each behaviour was also examined independently by repeated measures ANOVA across the discrete epochs. If this indicated an effect then separate one-way ANOVAs were performed for each time bin, with *post-hoc* Dunnett's tests to elucidate any drug effect. Food intake was examined by a one-way ANOVA with *post-hoc* Dunnett's test where appropriate. Animals were excluded from statistical analysis if their saline profile was atypical of the group insofar as they showed no inclination to feed from the food jars at any stage. Statistics are reported with the following format: [F(A) = B; $p < C$] where A is the degrees of freedom and B is the F value from the relevant ANOVA; C is the probability arising from *post-hoc* comparisons.

Randomisation. Each experiment was organised so that every animal received every treatment in randomised order, allowing animals to act as their own controls and reducing animal usage. Six animals were tested on any given test day, with 2 test days every week (Tuesday and Thursday) for the duration of the experiments. Animals tested on a Tuesday were always tested on a Tuesday, and the same applied to animals tested on Thursdays. Each of the 6 test cages was thus used for different animals on Tuesdays and Thursdays, but housed the same animal every Tuesday and Thursday. Thus not only did every animal receive every dose, but every test cage also saw each dose twice over the course of any given experiment, again in randomised order. Every treatment was given to at least one animal on each test day.

Experiment 1. Procedures differed from those outlined above in that it was the method of observation that was itself being assessed. On the day of testing animals were either scored as per the general method, or their session was recorded from a camera mounted high over the cage pens for later analysis. In place of drug treatment, the satiety-enhancing mechanism used was a 15 minute period of pre-feeding – in other words, introducing food 15 minutes before observation began. Six animals were tested in any given session, three coded live and three taped for video-analysis. Tapes were analysed, coding the animals into the same four distinct behavioural categories by timing and tallying the duration (to the nearest second) of each behavioural type within 5-minute time bins. For each method of observation the behaviour profiles of the pre-fed and non-pre-fed conditions were then examined for evidence of an advanced BSS, with animals used as their own controls. Statistical analysis was not

used. Randomisation differed from the general protocol in that only 3 of the test cages were set up with cameras to record sessions, thus each cage saw 4 different animals rather than 2, and each animal was exposed twice to each of two cages (pre-fed and non-pre-fed conditions in both a cage with a camera mount, and one without one). Observations were carried out 4 hours into the dark phase. One animal was excluded from analysis for having removed the barrier to the food hopper during one session.

Experiment 2. Procedures differed from the general method in that the treatments given were 3 different doses of *d*-fenfluramine plus a vehicle condition. Each animal was again used as its own control, and received 0.3 mg/kg, 1.0 mg/kg and 3.0 mg/kg *d*-fenfluramine plus a saline control dose in a randomised treatment order. Animals were under a light cycle such that observations began 4 hours into the dark phase, and dosing was carried out immediately before the food jars were presented. Animals were scored from live observations as described above.

Experiment 3. Procedures differed from the general method in that the treatments given were 3 different doses of sibutramine – 0.5 mg/kg, 1.67 mg/kg and 5.0 mg/kg – a 1.0 mg/kg *d*-fenfluramine positive control plus a vehicle condition. Each animal was again used as its own control, and received all four drug treatments and a saline treatment in a randomised treatment order. Dosing was such that animals received 2 injections; saline or an appropriate dose of sibutramine was given 30 minutes prior to the presentation of the feeding jars, and saline or *d*-fenfluramine was injected immediately before observation began. Animals only received one drug dose on any given day. Animals were under a light cycle such that observations began 4 hours into the dark phase and were scored from live observations as described above.

Experiment 4. Procedures differed from the general method in that the treatments used were 1.0 mg/kg of *d*-fenfluramine or a saline control dose. Administration of the doses was carried out 30 minutes prior to the presentation of the food jars. Animals were under a light cycle such that observations began 4 hours into the dark phase and were scored from live observations as described above.

4.3 Results

Experiment 1. **Figure 4.5.5** shows the behavioural profiles for animals observed by either live time sampling or coded from videotapes of sessions in the pre-fed or non-pre-fed conditions; results from all animals were averaged (video data) or summed to give a representation of how time in each 5-minute epoch was spent. A visual scan of the data indicates that the live-coding technique revealed a leftward shift in the behavioural satiety sequence in pre-fed animals. This was as expected from a satiety enhancing treatment, with an earlier onset of resting behaviour and corresponding reduction in feeding behaviour. A similar pattern can also be seen in the video-coded data. Live-coding was significantly less time consuming, and better suited the set-up already in place. Thus given it proved sufficient to highlight satiety enhancements and advancements of the BSS such as that with 15 minute pre-feeding it was decided that a live-coding methodology was sufficiently powerful for future investigations. **Figure 4.5.6*(a* and *b)*** illustrates the level of variation recorded with each of the two methods.

Experiment 2. **Figure 4.5.7** shows behavioural profiles for animals dosed with vehicle, 0.3, 1.0 or 3.0 mg/kg *d*-fenfluramine. The lowest dose of *d*-fenfluramine caused no variation from control conditions until 50 minutes into the observation period when cumulative feeding counts were significantly lower than observations under control conditions [$F(3,32) = 13.698$; $p < 0.05$]. Treatment with both 1.0 and 3.0 mg/kg *d*-fenfluramine advanced the offset of feeding, with feeding observations significantly lower than under control conditions after 25 and 10 minutes of observation respectively [$F(3,32) = 3.034$; $p < 0.05$ after 10 minutes for 3.0 mg/kg; $F(3,32) = 9.689$; $p < 0.01$ after 25 minutes for 1.0 mg/kg]; in all cases these differences were preserved over the 60 minute observation [$F(3,32) = 15.811$; $p < 0.01$ for 1.0 and 3.0 mg/kg *d*-fenfluramine; $p < 0.05$ for 0.3 mg/kg *d*-fenfluramine]. Activity observations were decreased by highest dose of *d*-fenfluramine compared to controls after 55 minutes [$F(3,32) = 4.522$; $p < 0.05$], and over the whole hour, but after other doses the levels of activity were always equivalent to those seen after saline dosing. No dose affected grooming at all. After animals had received 3.0 mg/kg *d*-fenfluramine the onset of resting was also significantly advanced with total resting observations significantly higher than controls after 10 minutes [$F(3,32) = 3.395$; $p < 0.05$], and remaining so thereafter [$F(3,32) = 18.913$; $p < 0.01$ after 60 minutes]. Under

conditions of treatment with 1.0 mg/kg *d*-fenfluramine, animals were observed resting more often late in the observation and this increase led to significantly more resting over the 60 minute observation period than seen under control conditions [$F(3,32) = 18.913$; $p < 0.05$]. The low dose of *d*-fenfluramine had no effect on the number of observations of resting behaviour. Drug treatment affected food intake in dose dependent manner [$F(3,32) = 22.065$; $p < 0.001$ for each drug dose], with all three doses significantly reducing one-hour food intake (**figure 4.5.10**).

Experiment 3. **Figure 4.5.8** shows behavioural profiles for animals dosed with vehicle or 0.5, 1.67 or 5.0 mg/kg sibutramine hydrochloride. The profile for 1.0 mg/kg *d*-fenfluramine is not shown. The 1.67 mg/kg and 5.0 mg/kg doses of sibutramine inhibited feeding early on, and significantly inhibited feeding throughout [after 5 minutes, $F(4,45) = 6.720$; $p < 0.01$ for both doses; after 60 minutes $F(4,45) = 13.230$; $p < 0.01$ for both doses] with a corresponding increase in resting (first seen after 5 minutes at 5.0 mg/kg [$F(4,45) = 8.61$; $p < 0.01$] and after 15 minutes with 1.67 mg/kg [$F(4,45) = 13.085$; $p < 0.05$]) which was likewise significant over the whole of the observation period [$F(4,45) = 8.867$; $p < 0.01$ for both doses]. The lowest dose, 0.5 mg/kg sibutramine, exhibited no quantifiable effect on feeding or resting behaviour at any time. 1.0 mg/kg *d*-fenfluramine inhibited feeding compared to controls after 25 minutes [$F(4,45) = 11.507$; $p < 0.05$], an effect maintained over 60 minutes [$F(4,45) = 13.230$; $p < 0.01$]; although animals were only first noticed to be resting more after 55 minutes when treated with this drug [$F(4,45) = 8.941$; $p < 0.05$] it did significantly increase total resting observations over the hour long observation period [$F(4,45) = 8.867$; $p < 0.05$]. Activity observations were unaffected by any treatment, while animals were observed grooming less often over the course of the 1 hour observation when dosed with 1.67 or 5.0 mg/kg sibutramine [$F(4,45) = 14.110$; $p < 0.05$], and more often after they were dosed with *d*-fenfluramine [$F(4,45) = 14.110$; $p < 0.01$], than when injected with saline. Food intake (**figure 4.5.10**) was unaffected by 0.5 mg/kg sibutramine but decreased in dose-dependent manner at higher doses [$F(4,45) = 13.943$; $p < 0.01$ for both 1.67 and 5.0 mg/kg sibutramine compared to saline], and with 1.0 mg/kg *d*-fenfluramine [$F(4,45) = 13.943$; $p < 0.01$].

Experiment 4. **Figure 4.5.9** illustrates the advancement of the BSS seen when dosing with 1.0 mg/kg *d*-fenfluramine 30 minutes before food presentation. Feeding was

significantly inhibited after 10 minutes compared to saline [$F(1,20) = 7.561; p < 0.05$] and remained significantly lower throughout the observation period [$F(1,20) = 17.428$; $p < 0.001$ after 60 minutes], whilst resting was significantly higher after *d*-fenfluramine administration right from the moment of food return [$F(1,20) = 9.543$; $p < 0.01$ after 5 minutes; $F(1,20) = 5.485$; $p < 0.001$ over the full 60 minutes]. Grooming was increased by *d*-fenfluramine over the early part of the observation period [$F(1,20) = 5.172$; $p < 0.05$ over the first 25 minutes] but not over the observation as a whole [$F(1,20) = 2.193$; NS] whilst activity was majorly decreased throughout [$F(1,20) = 25.534$; $p < 0.001$]. Food intake (**figure 4.5.10**) was significantly decreased by drug treatment [$F(1,20) = 7.778; p < 0.05$].

4.4 Discussion

It is clear from **figure 4.5.5** that the temporal shift in the BSS produced by a 15 minute bout of pre-feeding is picked up by the time-sampling method. At first glance the same would not be said of the video-analysed data as there appears to be little change in the onset of resting. However the incidence of feeding is reduced from the start in the pre-fed condition and resting observations are made a little earlier, so it appears that both methods did pick up the pre-feeding induced advance in the BSS. The traces in **figure 4.5.6** indicate that the degree of variation in data collected with either method is, by and large, comparable. If anything, the video-analysed data gave a slightly tighter data set. However had the time sampling method been carried forward, given it's more intensive and time-consuming nature the number of animals used would have been reduced and thus potentially opening the data up to more variation. Given this, and the fact that the laboratory had not previously done BSS observations and was lacking a number of features specified by Halford *et al.* (1998) as essential for BSS recording, a time-sampling methodology was unquestioningly more suited to the situation here. As such, since the time-sampling method detected a pre-feeding induced advancement it was decided to carry such a methodology forward through future experiments. All that said, standard time-sampling methods such as that used by Clifton *et al.* (1989) involve momentary sampling, scanning many cages and recording the behaviour electronically. The set-up here was sufficiently different to warrant further discussion, as it influenced the final decision to use time sampling and the method of sampling used. The cages used (**figures 4.5.1-4.5.3**) had been originally designed for use in social hierarchy experiments but were well suited to allowing full observation of the animals as required in a BSS paradigm from the point of view of live, time-sampled behavioural coding. Each cage also had a camera mount, but one that only allowed a single angle of observation, as opposed to the multi-angled view that Halford *et al.* consider essential for continual analysis. However, the cages were also built into surrounds that lined them up in two rows of three cages which, while having limited portability, would not line up *in situ* and thus scanning and momentary sampling as used by Clifton *et al.* (1989) was not possible. This limitation, the lack of an electronic recording in place, and the potential difficulties of acquiring and setting such a system up, informed the decision to use a sampling period of five seconds, recording the predominant behaviour shown in that time by

marking one of four behavioural categories on a timesheet. The final difference from established paradigms is that rats were pair housed when not undergoing observation or habituation. This was a concession made to the Home Office who were not happy with single housing of animals for the length of the trials, especially given the observation cages were not the home cages. This limitation actually increased the length of the experiments as it became necessary to allow 24 hours isolation before the experiments to try to reduce the impact of any isolation stress on the BSS observations (stress being a known confounding factor in CNS pharmacology; Stanford, 1996). Furthermore such isolation stress could have been a significant factor in the non-responding of some animals, where they would just sit in the corner of the observation cage and show no interest in the presented food, even after significant food deprivation (6 hours; 24 hours during initial habituation) and under control conditions.

This is clearly far from an ideal method. Altmann (1974) suggested that for true accuracy in determining the frequency of behaviours the sampling interval should be shorter than the shortest duration of any behaviour and with rats this is frequently under one second for general behaviours such as sniffing or rearing. The inference is thus that a time sampling method can never give a true estimate. Furthermore, it could be argued that a longer observation per sample increases observer bias as the coding requires a conscious choice of dominant behaviour from a number of observed behaviours as opposed to an almost instantaneous judgement made on a momentary observation. The counter-argument is that a longer observation period per sample should help counter "event over state" bias with longer lasting, state-like, behaviours now given more weight. The risk that the balance may swing the other way, overly in favour of state-like behaviours, is a valid concern; however both feeding and resting are arguably state-like behaviours, at least when examined as described here, and activity is a catch-all category for all behaviours that are not covered by the other categories. Since active behaviours will often give way to other active behaviours a sequence of active behaviours could also be said to be "state-like" activity and thus it is the grooming observations that are most likely to be affected by this change. A comparison of the time-sampled data and rudimentarily video-analysed data in **figure 4.5.5** suggests that observations of grooming are indeed affected and under-recorded with the time-sampling method. However the base temporal shift in the BSS is clearly

visible and, with the shift from feeding behaviour to resting behaviour being of prime importance, the technique was sufficient to measure the key feature. Additionally the methodology carried forward and used from this point on does carry one distinct advantage over previously characterised methods. By using animals as their own controls within each discrete experiment, animal usage is reduced and the degree of error by interpreting results across different animals is also minimised. Additional rationalisation for the approach and methodology used here is discussed briefly later on [chapter 8.0].

Moving on to look at the drug trials, the first stage, whilst awaiting the arrival of the sibutramine donation, was to chose an appropriate dose of *d*-fenfluramine to serve as a comparison to sibutramine in later circadian trials. Both racemic fenfluramine and the *d*-isomer used here have been extensively studied using the BSS (*e.g.* Halford *et al.*, 1995, 1998; McGuirk *et al.*, 1992*a*; Montgomery and Willner, 1988; Willner *et al.*, 1990). The majority of work supports the observation that this drug advances the satiety sequence, though some suggest it disrupts resting by encouraging activity (Montgomery and Willner, 1988; Willner *et al.*, 1990). The present results support the former conclusion; at the two higher doses tested, 1.0 and 3.0 mg/kg, *d*-fenfluramine caused a temporal shift in the BSS profile, advancing the offset of feeding in the first half of the observation period and promoting the onset of resting such that animals were observed resting more over the length of the observation period. The lowest dose of *d*-fenfluramine (0.3 mg/kg) produced no change in resting, while the effect on feeding was small and observed late in the observation period. At the highest dose tested the offset of feeding behaviour was advanced so that animals were observed feeding significantly less after 10 minutes into the observation when given the 3.0 mg/kg dose than when given saline. A similar decrease in feeding was seen after 25 minutes in observations carried out after dosing with 1.0 mg/kg *d*-fenfluramine. A corresponding advance in the onset of resting behaviour was observed after the 3.0 mg/kg dose, with resting observations scored more often than under saline conditions after 10 minutes, and total observations in the hour-long observation remained lower than when these same animals were dosed with saline. There was a trend of increased resting after dosing with 1.0 mg/kg *d*-fenfluramine too, and instances of resting were raised in the latter period of the observations compared to controls leading to a significantly higher number of resting observations over the 60 minute test period.

Perhaps more relevant than the base statistics is the general profile in these cases; associated behavioural symptoms not revealed by the profiles shown in **figure 4.5.7** may also be a factor. Firstly it is difficult to investigate temporal shifts in the offset of feeding if there is not a significant initial feeding response. After being given 3.0 mg/kg *d*-fenfluramine the animals' initial feeding response was almost completely ablated, with virtually no feeding seen beyond 5 minutes. While it could be argued that this is indicative of a strong satiating effect and an extreme advance in the satiety sequence, the magnitude of the drug effect could prove unhelpful in identifying underlying changes due to circadian rhythm in later experiments. This dose also caused animals to exhibit a low, outstretched body posture when moving and inhibited activity as a whole over the hour-long observation. This inhibition is consistent with reports of racemic fenfluramine being sedative at doses lower than 3.0 mg/kg (*e.g.* Aulakh *et al.*, 1988). The *d*-isomer has itself been shown to decrease locomotor activity and increase the energy cost of muscular effort (Even and Nicolaidis, 1986) at 7.0 mg/kg; though this is much higher than the dosages used in the present work, a smaller effect may contribute to some degree to the behavioural profile seen here with 3.0 mg/kg *d*-fenfluramine. This apparent sedation, regardless of the mechanism behind it, could have interfered with recording of circadian variation in satiety enhancement had the 3.0 mg/kg dose been selected for that work. Therefore, given that a noticeable shift in the BSS profile is seen with 1.0 mg/kg *d*-fenfluramine, a dose considered equipotent with the ED_{50} of sibutramine for 2-hour inhibition of food intake and at which *d*-fenfluramine has been seen to advance the BSS before (Halford *et al.*, 1995, 1998), this intermediate dose was chosen as the most appropriate to use as a comparison with sibutramine in later circadian trials.

Figure 4.5.8 shows the profiles from three log-separated doses of sibutramine and saline control. Again, as with the *d*-fenfluramine results above, the aim of the experiment was to select an appropriate dose of sibutramine to use in the forthcoming circadian trials. Here the lowest dose tested produced no significant change from the saline condition. On the contrary the medium (1.67 mg/kg) and high (5.0 mg/kg) doses of sibutramine both exhibited a profound effect on the BSS profiles. Under both treatments the instance of feeding was significantly reduced throughout the observation period when compared to the observations after saline injections. Both these drug treatments also increased the observations of resting behaviour over the

hour long test session. The 1.67 mg/kg dose accelerated the onset of resting, with animals observed resting significantly more often after 15 minutes compared to when they were injected with saline. By contrast, the 5.0 mg/kg dose increased resting throughout the observation period, with a high level of resting observations even in the first 5 minutes after food was returned. Both these doses of sibutramine also reduced the observation of grooming behaviour over the course of the hour-long observation period, without affecting the instance of activity counts. In contrast, the 1.0 mg/kg *d*-fenfluramine dose selected after the experiments described above actually caused an increase in grooming observations over the hour. This drug also increased total resting observations, statistically significant only after 55 minutes despite a trend of increased resting throughout when compared to controls, as well as advancing the offset of feeding behaviour, with significantly fewer observations of feeding after 25 minutes than under control conditions.

Looking at the behavioural profiles, the high dose of sibutramine did not so much advance the satiety sequence as ablate feeding altogether. The initially high frequency of resting observations is typical of a sedative effect, although activity scores remain unaffected. It is possible that at 5.0 mg/kg sibutramine is satiating the animals enough to prevent any feeding, even after 6 hours of food deprivation, 4 hours of which was at a time in the light: dark cycle when rats tend to consume the majority of their daily intake. Regardless of whether this lack of intake is due to sedation or satiety it was clear that with no intake present at this circadian time, it was not feasible to study this high dose of sibutramine for differing effects on the BSS at times when animals would naturally feed less. Likewise, the low dose of sibutramine caused no detectable change in the BSS profile and was not a viable candidate for future work. The 1.67 mg/kg dose on the other hand provided a clearly visible shift in the BSS whilst maintaining a definite feeding response, albeit a very much reduced one. This dose was also effective at reducing food intake, and is close to a previously reported ED_{50} dose of 2.0 mg/kg for 2-hour inhibition of food intake (Halford *et al.*, 1995). As such this dose of 1.67 mg/kg sibutramine was selected as the dose to be used in the circadian experiments described below. Moreover in this experiment the 1.67 mg/kg dose of sibutramine and the 1.0 mg/kg dose of *d*-fenfluramine were equipotent in reducing food intake (as shown in **figure 4.5.10**) and thus a good matching pair to use in studies spread across the circadian cycle..

With the doses to be used selected, and the methodology settled upon, the final variable to decide upon before progressing onto the circadian studies was the dose-test interval. To this point, sibutramine had been administered 30 minutes prior to the return of food and the beginning of observations, time enough for the drug to be metabolised to its active forms, whilst previous dosing with *d*-fenfluramine had been done immediately prior to the start of observations. Although the double-dosing used in *Experiment 3* above was one way of dealing with this dichotomy it still left the possibility of an injection-induced effect in the early stages of the experiment, which may have a masking effect on early effects of the drugs. To see if this could be avoided, animals were tested with 1.0 mg/kg *d*-fenfluramine given 30 minutes before the re-introduction of food to investigate whether this procedural change would still allow detection of the advance in the BSS caused by this drug dose and simultaneously remove any immediate reaction to injection from the observations made. The resulting BSS profile can be seen in **Figure 4.5.9**. This change appears to increase the resting observations, but there is still a robust feeding response and an earlier cessation of feeding than seen with saline-treated animals, and was therefore accepted into the procedure used from here on.

In summary, the experiments described above allowed for selection of a protocol and drug doses to be used in the circadian studies that form chapter 7 of this thesis. The doses selected were 1.0 mg/kg *d*-fenfluramine and 1.67 mg/kg sibutramine, to be administered 30 minutes prior to the return of food. Observations were then made by a time sampling method and coded live for 60 minutes following the return of food. The doses determined here were also used for circadian locomotor work [chapter 5, below].

4.5 Figures for Chapter 4

Figure 4.5.1 An example of the cages used for behavioural satiety sequence (BSS) observations, shown *in situ*

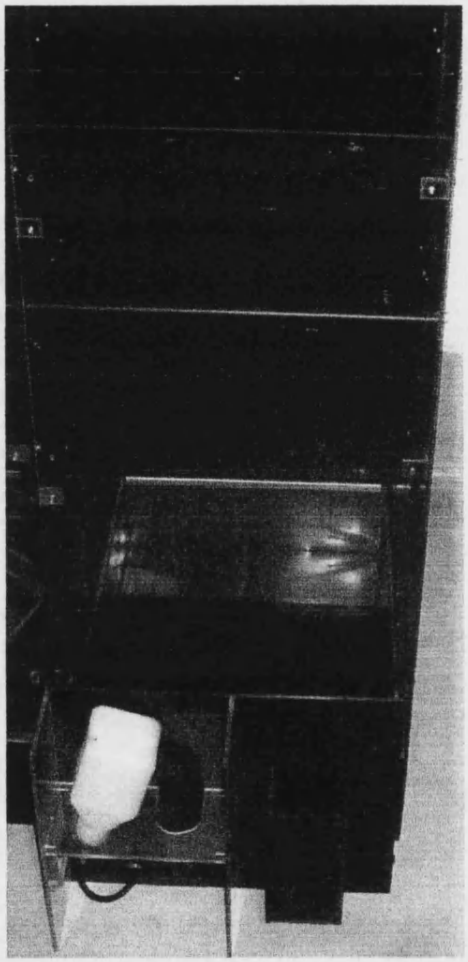


Figure 4.5.2 Closer detail on a BSS observation cage tray, showing the food hopper and water provision

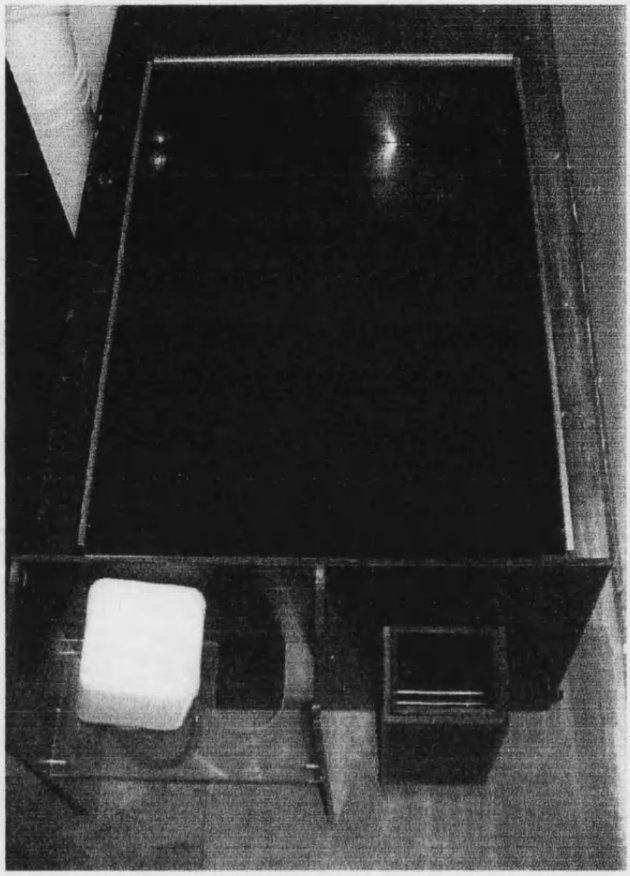


Figure 4.5.3 Photograph showing the sliding plate mechanism by which access to food was restricted in the run up to, and during, BSS observations. Access to water was not similarly restricted

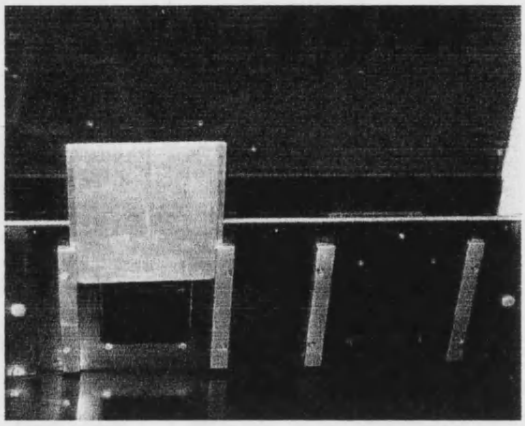
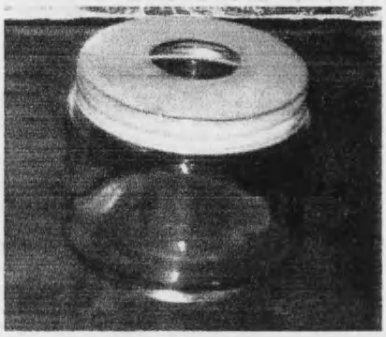


Figure 4.5.4 Photographs illustrating the feeding jars in which ground food was presented for BSS, and circadian locomotor activity, experiments



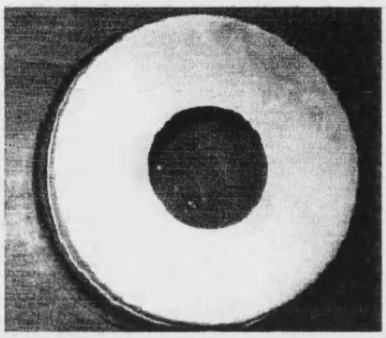


Figure 4.5.5 Behavioural profiles from BSS observations by two methods; animals were either pre-fed (B, D) or presented with food immediately prior to observations.

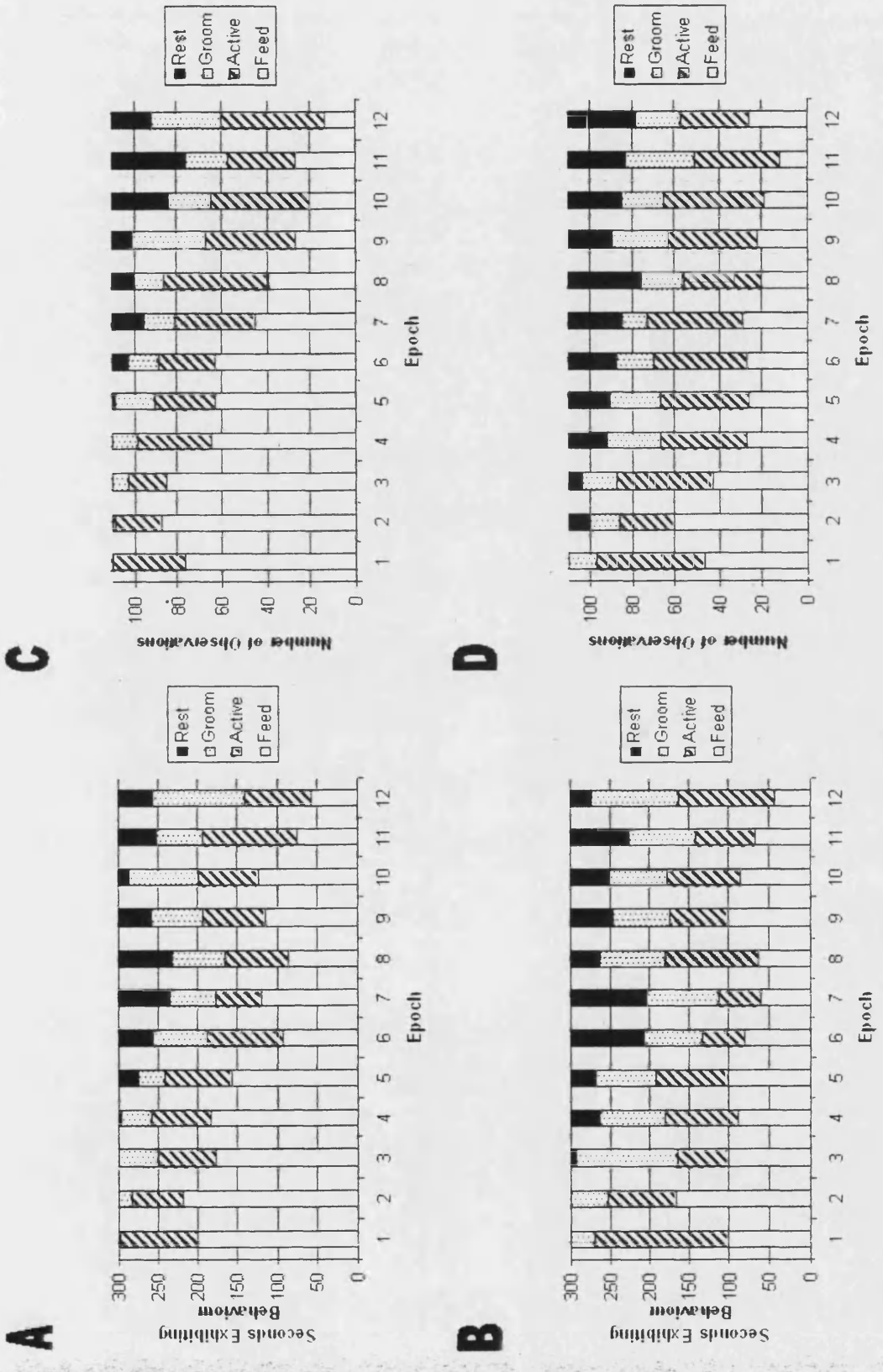


Figure 4.5.5

Behavioural profiles generated in the BSS by analysis of videotapes (**A**, **B**) or live coded data (**C**, **D**). Food was returned either at the beginning of the first epoch (**A**, **C**) or 15 minutes before the start of the observation period (**B**, **D**). $n = 11$ in every case, and the same animals were used under each of the 4 conditions. Each epoch was 5 minutes long. The values for *Seconds Showing Behaviour* are averages across the 12 animals whilst those for the *Number of Observations* are aggregates of scores for all 12 animals during any given epoch. Both methods indicate a leftward shift in the offset of feeding and the onset of resting consistent with an advance in the BSS.

Figure 4.5.6*a* Cumulative measures of behaviour coded from videotape over 60 minutes of BSS studies.

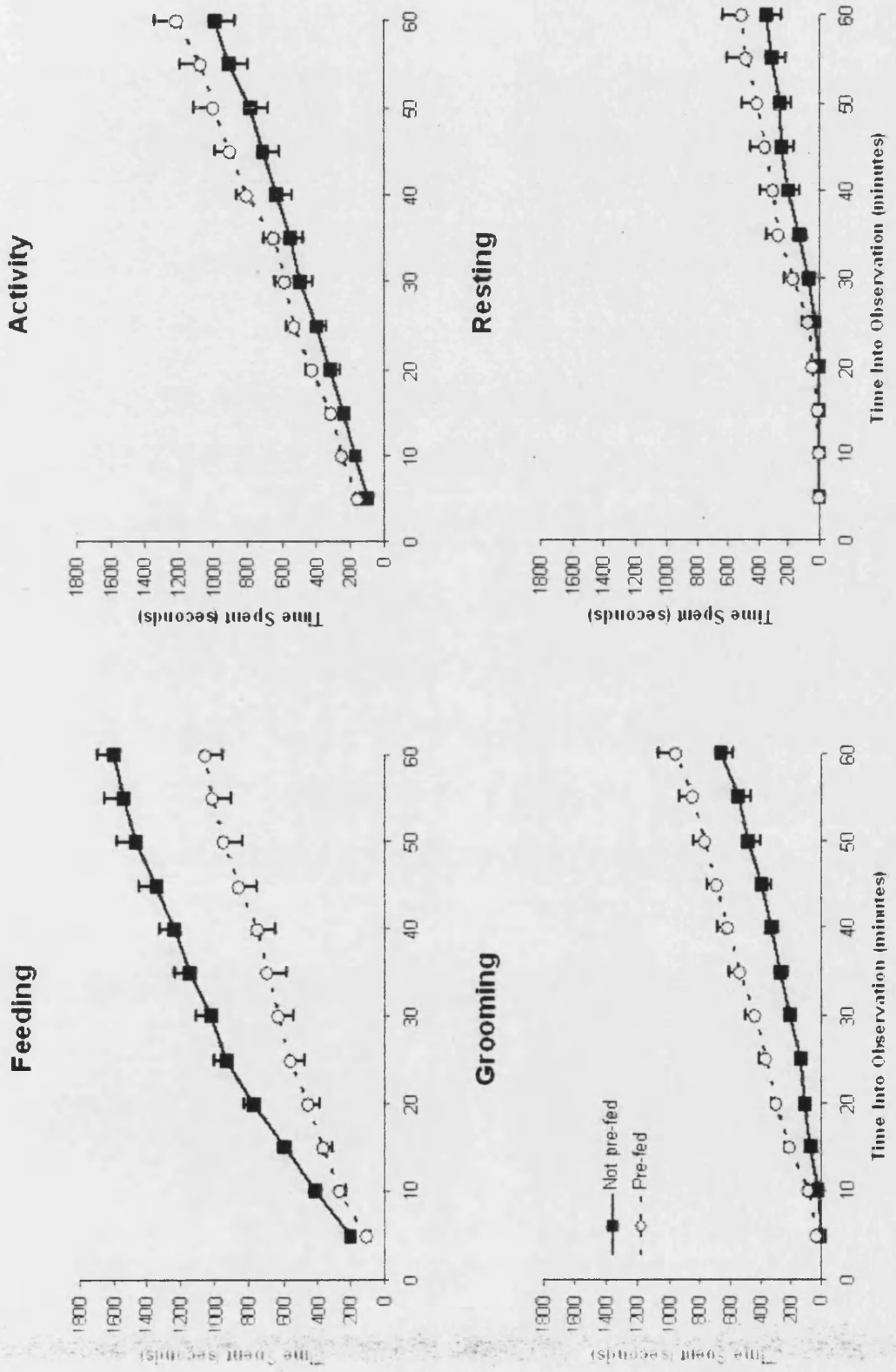


Figure 4.5.6*b* Cumulative measures of behaviour coded by live time sampling over 60 minutes of BSS studies.

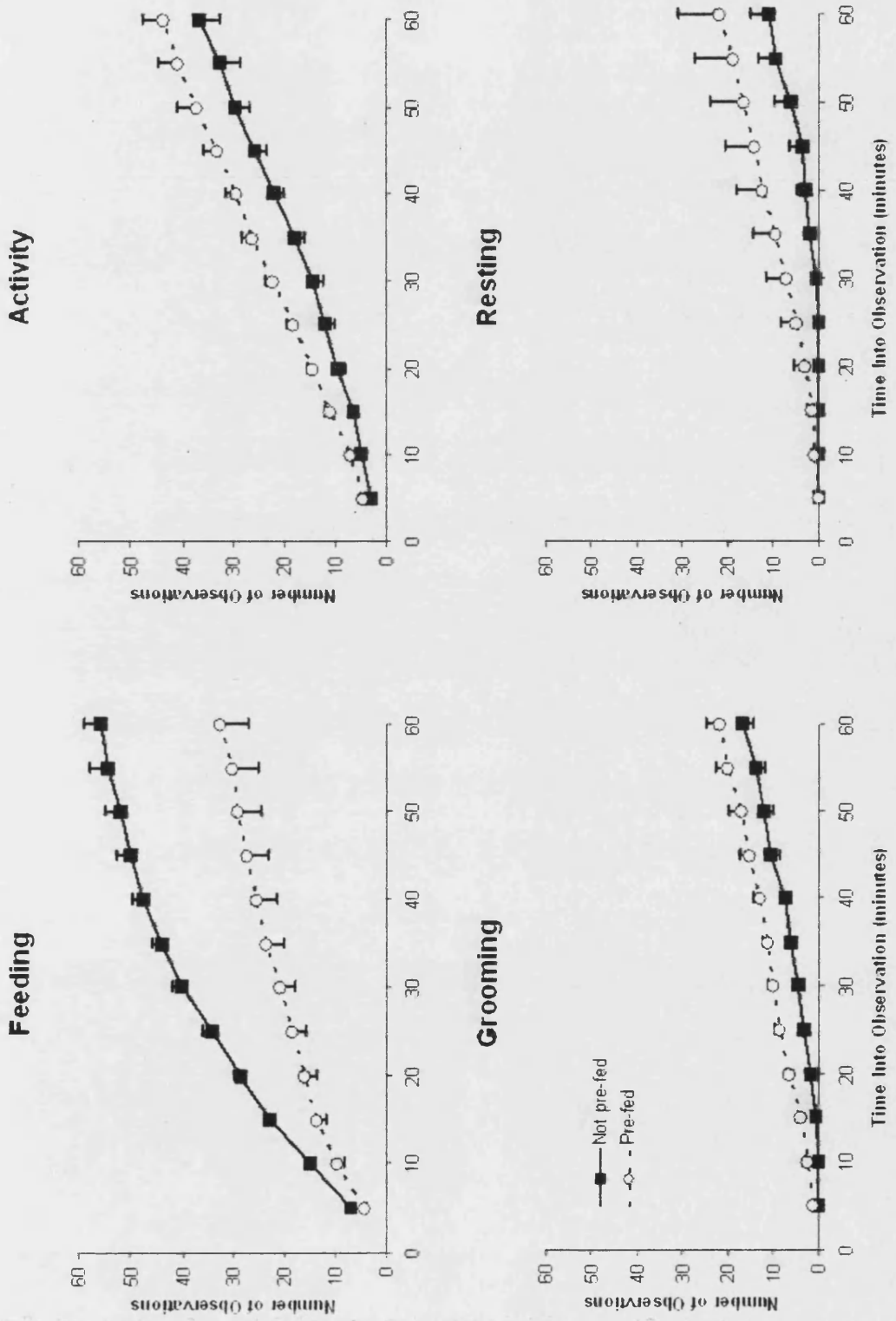


Figure 4.5.6

Cumulative time-plots for all 4 coded behaviours indicating the variation in the data under both pre-fed and non-pre-fed conditions when scored from videotaped sessions (*a*) or time sampled (*b*). Data are means ± standard error, $n = 11$.

Figure 4.5.7 Behavioural profiles from BSS observations with 3 doses of *d*-fenfluramine and saline.

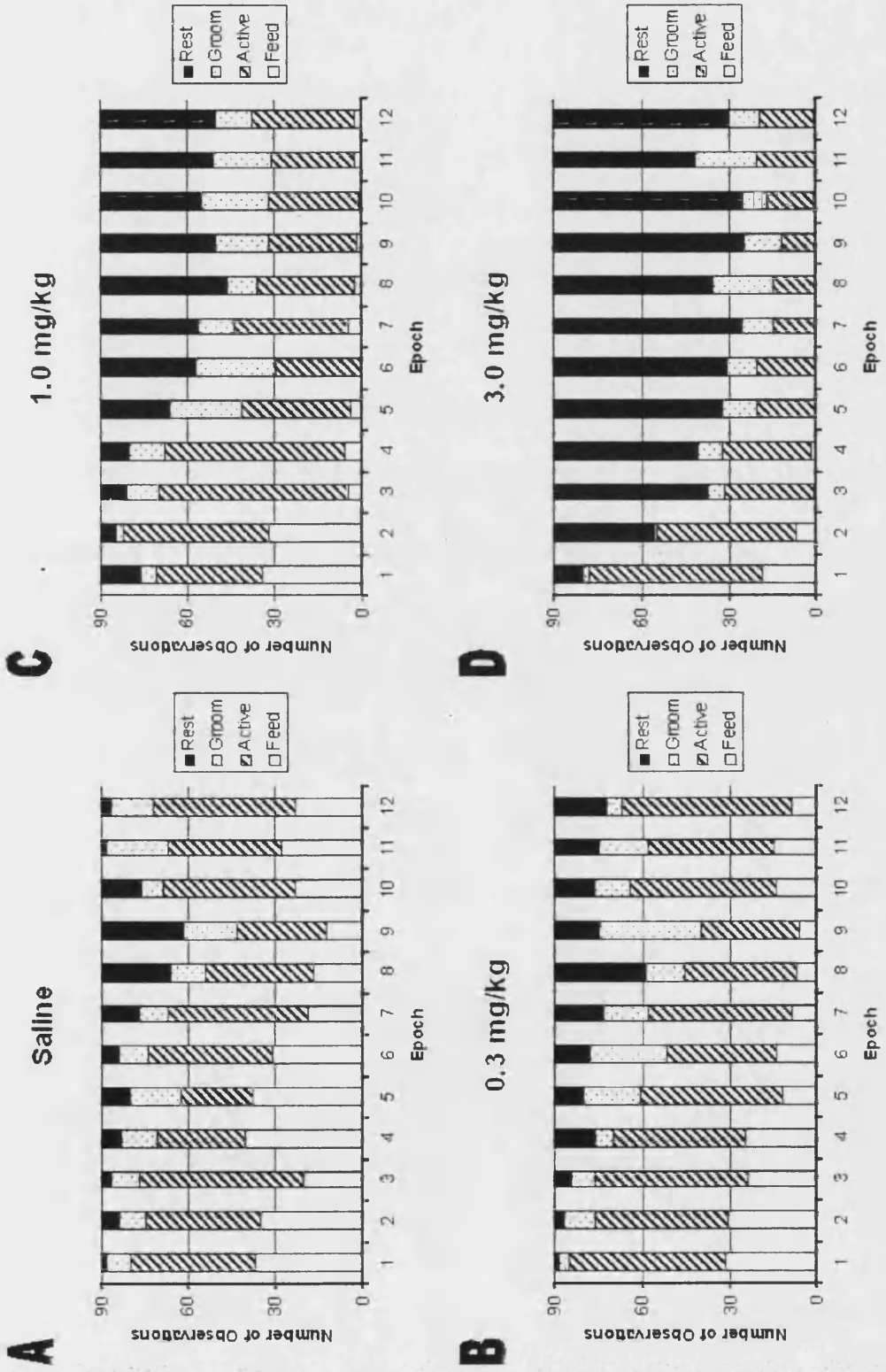


Figure 4.5.7

Behavioural profiles generated after administration of three different doses of *d*-fenfluramine or saline 4 hours into the dark phase. Observations are aggregates of all scores for each animal in any given 5-minute epoch. A clear advance in the BSS is seen with both 1.0 and 3.0 mg/kg *d*-fenfluramine but the lowest dose of 0.3 mg/kg had no effect. $n = 9$ in every case, and the same animals were used for all 4 treatments.

Figure 4.5.8 Behavioural profiles from BSS observations with 3 doses of sibutramine and saline.

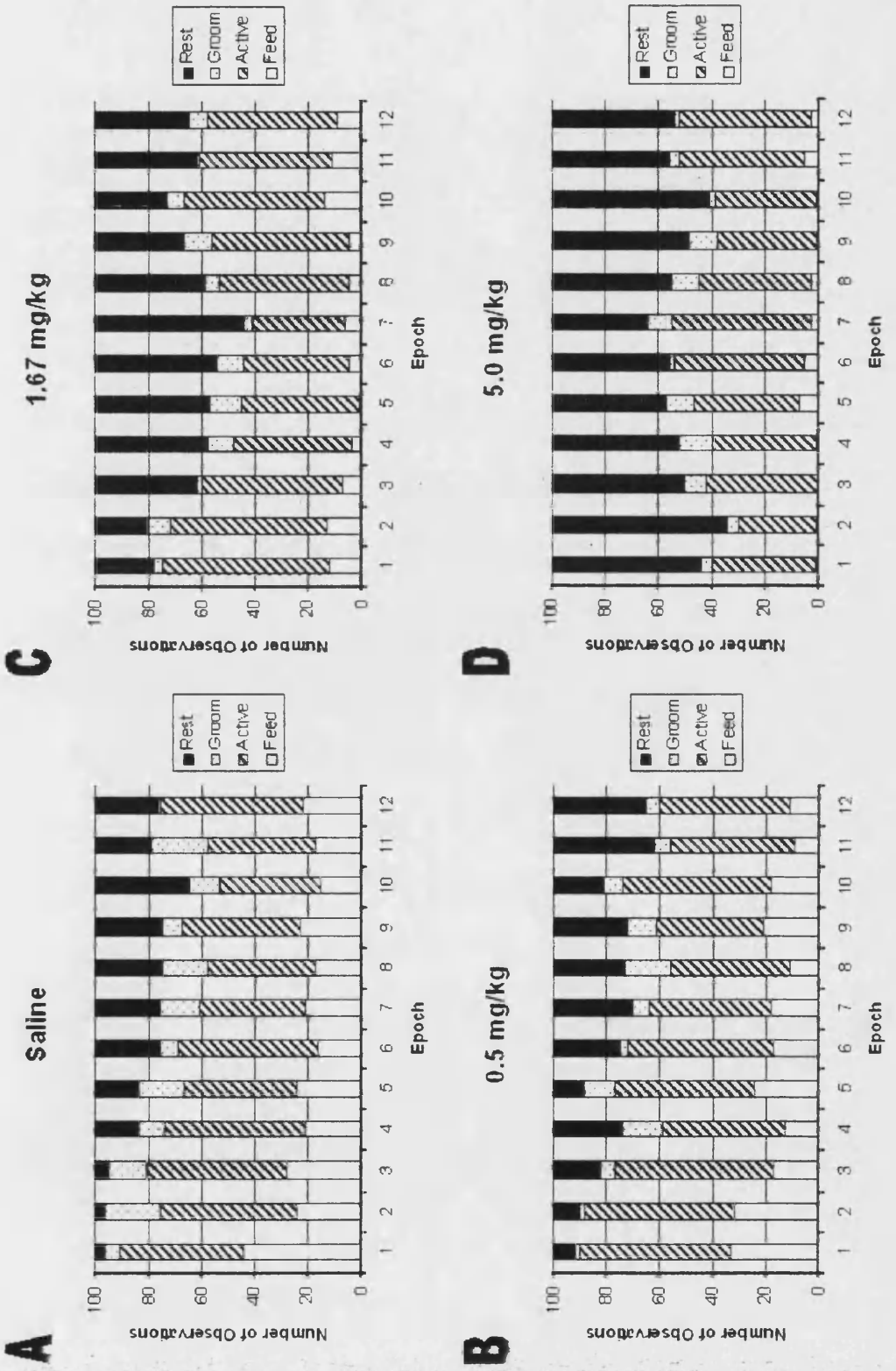
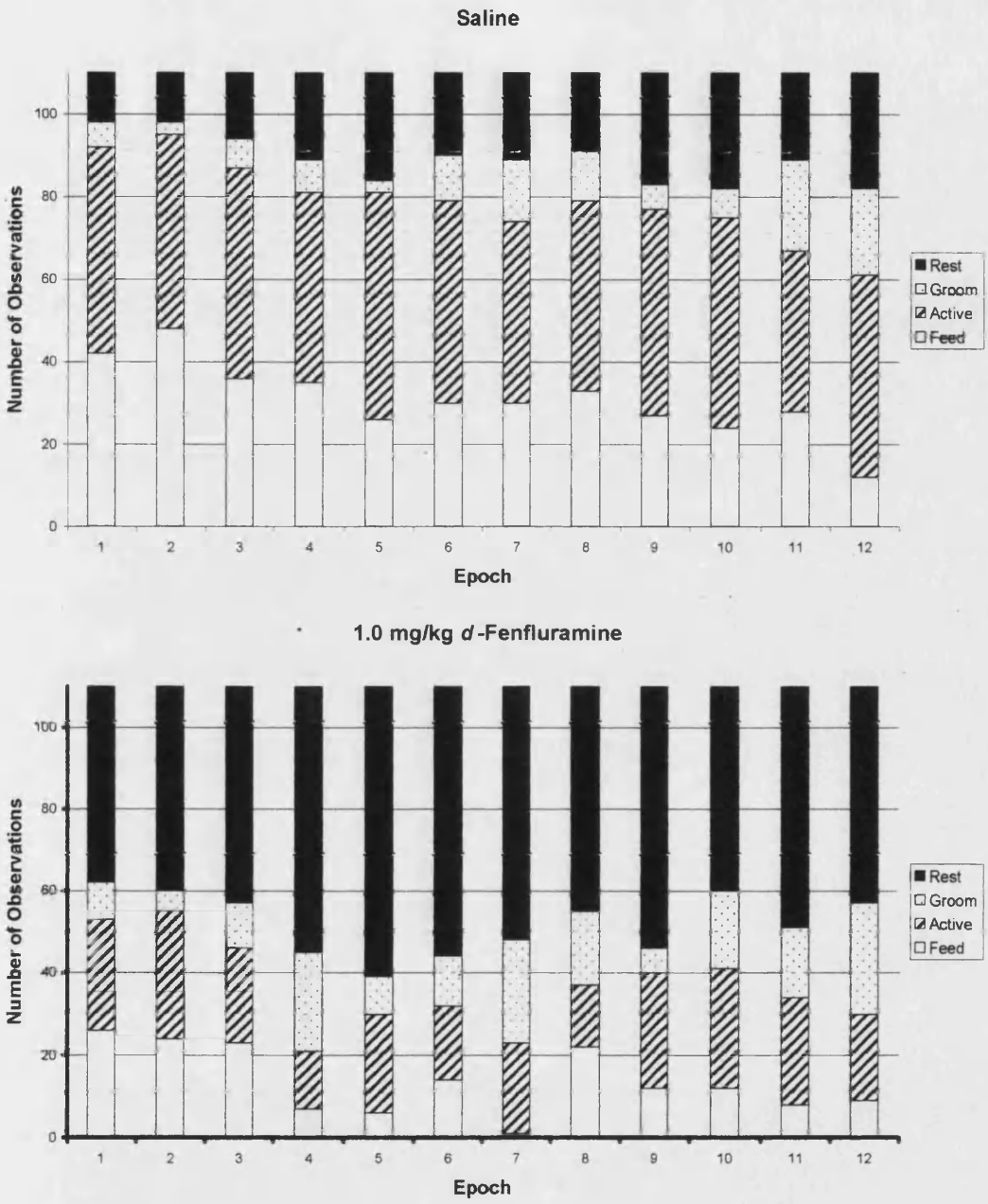


Figure 4.5.8

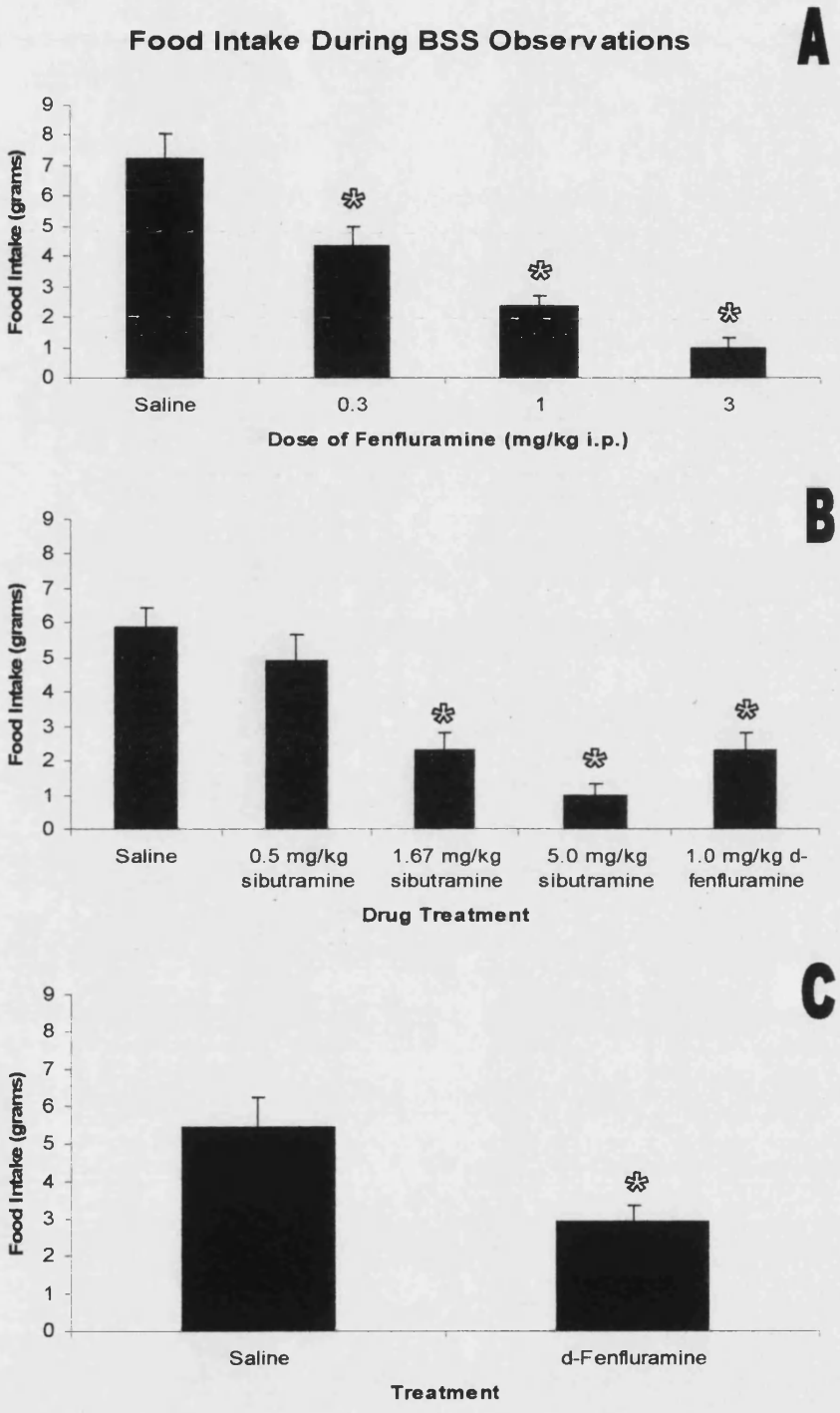
Behavioural profiles generated after administration of three different doses of sibutramine or saline 30 minutes prior to food return (4 hours into the dark phase). Observations are aggregates of all scores for each animal in any given 5-minute epoch. A clear advance in the BSS is seen with both 1.67 and 5.0 mg/kg sibutramine but the lowest dose of 0.5 mg/kg had no effect. *n=10* in every case, and the same animals were used with all 4 treatments. A profile generated with 1.0 mg/kg *d*-fenfluramine is not shown.

Figure 4.5.9 Behavioural profiles from BSS observations carried out with 1.0 mg/kg *d*-fenfluramine or saline given 30 minutes prior to the start of observation



Behavioural profiles generated after administration of *d*-fenfluramine or saline 30 minutes prior to food return (4 hours into the dark phase). Observations are aggregates of all scores for each animal in any given 5-minute epoch. A clear advance in the BSS is seen compared to saline. $n = 11$.

Figure 4.5.10 Food intake as measured in *experiment 2* (*a*), *experiment 3* (*b*) or *experiment 4* (*c*)



❋ $p < 0.05$ compared with saline treatment.

5.0 The Effect of Sibutramine and *d*-Fenfluramine on Locomotor Activity, Measured at Different Times Spread Across the Circadian Cycle

5.1 Introduction

The circadian influence on activity and other biological rhythms is well documented, as reviewed above [section 3.3]. Alongside the investigation into the effects of circadian rhythms on behavioural satiety sequence observations with the centrally acting anorectics sibutramine and *d*-fenfluramine [chapter 7, below], parallel investigations were carried out to examine any circadian influence on locomotor responses to these drugs, and to examine how this might impact on the interpretation of the BSS observations.

Both sibutramine and fenfluramine have been examined previously for effects on locomotor drive, with sibutramine exhibiting no effect (Rowley *et al.*, 2000) and with (*d*-)fenfluramine showing signs of sedative activity (Aulakh *et al.*, 1988; Callaway *et al.*, 1993 Ziance *et al.*, 1972) that is independent of its primary pharmacological mechanism of precipitating serotonin release (Callaway *et al.*, 1993). This effect may be in part, or in full, the result of an increase in the energy cost of locomotion induced by *d*-fenfluramine (Even and Nicolaidis, 1986). However neither sibutramine nor indeed (*d*-)fenfluramine has been previously investigated for locomotor effects with full regard to the possible impact of circadian rhythm. While circadian impact on therapy with (*d*-)fenfluramine has been reported in the clinic (Katz *et al.*, 1999) and (*d*-)fenfluramine has been used as a tool in other circadian serotonergic studies (*e.g.* Biello and Dafters, 2001), the effect on locomotor activity has not been looked at across the circadian cycle. Understandably given the lack of locomotor effects previously observed with sibutramine (Rowley *et al.*, 2000) this drug has not been further investigated for circadian influences. The nature of circadian variation is such that not only do basal levels of activity change but so do neurotransmitter levels, and many other functions – including how an organism responds to, or metabolises, pharmacotherapy (*e.g.* Martin and Redfern, 1997; Labreque and Bélanger, 1991; section 3.3). Drugs like sibutramine and *d*-fenfluramine exert effects via 5-HT – a neurotransmitter which exhibits a highly variable circadian rhythm (Martin and

Redfern, 1997) – and are therefore effectively, *in vivo*, working against a very different neurochemical backdrop depending on when they are administered. As such it is plausible that while they might have little or no acute effect on locomotor behaviour when given at one time of day, they may exert more noticeable or significant influence at other times. The general field of circadian influence is reviewed above [section 3.3], both with respect to 5-HT rhythmicity and to locomotor activity.

It is also important to consider for a moment the general control of motor activity, and specifically the role of 5-HT in motor control. For a long while 5-HT was generally considered to have a negative action on motor activity, inhibiting its expression. The rationale for this was that 5-HT antagonised dopaminergic increases in activity, such as those produced with amphetamine, and studies showed a trend for increased levels of activity in 5-HT-deficient animals (see Gerson and Baldessarini (1980) for a period-appropriate review). These views were supported by the fact that in rats the period of greatest activity also corresponds to the period of lowest brain 5-HT. levels (see review by Martin and Redfern (1997) and section 3.3). More recently however, while the fact that 5-HT can have an inhibitory effect has not been questioned (for example see Di Matteo *et al.*, 2001, a review of tonic inhibitory control by 5-HT_{2C} receptors over dopaminergic systems), it has become clear that the full role of 5-HT in locomotor control is a lot more complicated. Additionally it is now known that 5-HT release, usage and metabolism are greatest during the active phase [see section 3.3]. Some direct serotonergic agonists, particularly those primarily active at 5-HT_{1B} receptors, and some 5-HT releasing agents (*e.g.* 3,4-methylenedioxy-metamphetamine; MDMA) can have a potentiating effect on activity (Geyer, 1996), whether by modulating mesolimbic dopaminergic control in the venteral tegmental area or nucleus accumbens (reviewed by Mylecharane, 1996), or through other pathways such as the 5-HT_4 receptor activity in the hippocampus described by Takahashi *et al.* (2002) where the mechanisms underlying the effects on activity are yet unknown. Interestingly, (*d*-)fenfluramine is an exception, a 5-HT releasing agent which suppresses rather than increases locomotor activity (Aulakh *et al.*, 1988; Callaway *et al.*, 1993; Geyer, 1996; Ziance *et al.*, 1972). These effects are independent of 5-HT release (Callaway et al., 1993) and may be due to the drug increasing the energy cost

of locomotor activity (Even and Nicolaidis, 1986). For a more complete review of serotonergic influences on motor control see Geyer (1996).

The previous body of work on sibutramine and the BSS suggests that its anorectic effects are due to an enhancement of satiety (Halford *et al.*, 1995, 1998) rather than behavioural activation (as seen with amphetamine for example; Halford *et al.*, 1995, 1998), sedation (as with MK-212; Halford *et al.*, 1996*a*, 1998) or making food unpalatable (like quinine does, Antin *et al.*, 1975). My own initial results from BSS experiments looking at sibutramine would support this assertion [chapters 4 and 7]. However, with the neurochemical background so variable over the circadian cycle and the potential impact of circadian variation in hepatic metabolism of sibutramine to its active metabolites [section 3.4, above], it was considered interesting, and pertinent, to investigate whether circadian variation had an impact on the effects of both sibutramine and *d*-fenfluramine on locomotor function in parallel with investigating any circadian influence under a BSS paradigm [chapter 7]. The existing research on acute racemic fenfluramine in the BSS is .somewhat conflicting; although the prevailing view is that acute fenfluramine advances the BSS (*e.g.* Halford *et al.*, 1995, 1998) there are some reports of a disruption of the sequence due to a displacement of resting behaviour to active behaviours (including locomotor activity and grooming) after the offset of feeding (Montgomery and Willner, 1988; Willner *et al.*, 1990; McGuirk *et al.*, 1992*a*). Couple this to the reports of fenfluramine as a locomotor depressant or sedative agent (Aulakh *et al.*, 1988; Callaway *et al.*, 1993, Ziance *et al.*, 1972) and a mixed picture emerges of the effects of fenfluramine on locomotor activity, and that before examining any potential circadian influence.

Given that the BSS paradigm used [chapters 4 and 7] did not itself explicitly record locomotor activity, the experiments described below were carried out to quantify locomotor effects of drug treatment with sibutramine and *d*-fenfluramine across the circadian cycle in animals subjected to similar food deprivation schedules used in the BSS studies.

5.2 Materials and Methods

Animals Adult male Wistar rats (Bath University strain; 150-280g at the start of experiments) were used in all experiments, unless otherwise noted. Animals were kept at 21°C (+/- 1°C) under a 12:12 light/dark cycle. Rats were housed in pairs with free access to food (CRM(E) pellets; Special Diets Services, Witham, UK) and water for 10 days, then singly housed with *ad libitum* access to food and water for a further week before locomotor testing began. Dark-phase illumination was provided by a single 15 Watt red light bulb on a mobile fitting so as to allow examination of all cages.

Drugs. Sibutramine hydrochloride monohydrate and *d*-fenfluramine hydrochloride (RBI, donated by RenaSci) were both dissolved in 0.9% saline (Fresenius Kabi, Warrington, UK). Fresh drug solutions were made up each day and all injections were with 2 ml/kg of the drug solution or vehicle into the peritoneum, such that doses outlined in the individual experiments below were administered.

General Procedure. Animals were housed individually in transparent cages, which sat inside a 7x4 infra-red beam locomotor monitoring grid (**figures 5.5.1** to **5.5.4**). Two and a half weeks were allowed for animals to habituate to the light cycle.

Animals were food deprived 6 hours before the testing period, matching the deprivation schedule used for the BSS [chapters 4 and 7]. Animals were dosed 30 minutes before food was re-introduced in the form of ground up pellets in a glass feeding jar (**figure 4.5.4**). These jars had metal lids with 4 cm diameter round holes cut in them to allow access to the food inside and animals were habituated to feeding on the ground pellets at least once before test runs began. Once dosed, animals were free from human interaction until the following morning.

Locomotor activity records were amassed for 8 hours following the withdrawal of food (*i.e.* 6 hours during food deprivation and 2 hours after food re-introduction) with the period of particular interest being the hour after food was returned. Animals were used as their own controls and were dosed with each treatment once in a randomised order. Every animal was tested three times over the course of 3 days. The difference

in weight of the food jars between their return and their withdrawal the next morning was recorded in each case.

Statistical Analysis. At each circadian time, one way ANOVA tests were used on cumulative data for 5 minute epochs through the 60 minute period post food re-introduction with *post-hoc* Dunnett's tests used where appropriate. Collated data from all circadian times were examined by two-way ANOVA for the effect of drug treatment (*post-hoc* Dunnett's test) and circadian time (*post-hoc* Bonferroni test) on total locomotor activity in the hour following the return of food. The effect of circadian time was then further investigated on a treatment-specific basis, with one-way ANOVAs used on cumulative data with *post-hoc* Bonferroni tests used to elucidate any effect of circadian time. Statistics are reported with the following format: [F(A) = B; $p < C$] where A is the degrees of freedom and B is the F value from the relevant ANOVA; C is the probability arising from the ANOVA or *post-hoc* comparison as applicable.

Randomisation. Treatments were randomised such that every animal received every treatment, in random orders. With 12 animals in 12 cages and three treatments, 4 animals received each treatment each day.

Six separate experiments of this nature were carried out such that discrete groups of animals were tested at Circadian Times (CT) 1, 5, 9, 13, 17 and 21 where CT0 was lights-on, and CT12 was lights-off. Light cycles were set accordingly for each time point so that food deprivation would be at 10.00 hours GMT, and re-introduction at 16.00 hours GMT – so for example, when testing at CT5, lights were set to be on from 11.00-23.00 GMT, with food re-introduced at 16.00 GMT.

Experiments at CT1, CT5 and CT17 differed from the above procedure. In these cases one set of animals was used for saline and sibutramine conditions as above but received 1.0 mg/kg *d*-fenfluramine immediately prior to food re-introduction. This differed from the prior dosing regime of 30 minutes before food return used in every other case. Subsequently separate sets of animals were used to investigate the effects of 1.0 mg/kg *d*-fenfluramine when given 30 minutes prior to food re-introduction at each of these circadian times. These latter data are presented here and related to the

relevant saline and sibutramine results. Thus at Circadian Times 1, 5 and 17 animals were not used as their own controls in the case of *d*-fenfluramine treatment.

5.3 Results

For simplicity, the separate experiments have been re-ordered such that they are reported in order of advancing CT, from CT1 (early light phase) to CT21 (late dark phase). The results outlined below are summarised in **figure 5.5.5** and shown individually in **figures 5.5.5a-f**.

CT1. Following food re-introduction, neither drug exhibited any initial effect – neither stimulation nor depression – on locomotor activity as measured by cumulative beam breaks. Animals treated with *d*-fenfluramine never differed from controls. After 30 minutes of access to the feeding jars there were signs that under sibutramine conditions animals were exhibiting less total activity than controls [$F(2,33) = 3.467$; $p < 0.05$]. However by 55 minutes after the re-introduction of food these earlier differences were non-significant.

CT5. A significant decrease in locomotor activity was noted from the moment that the food jars were presented in animals treated with *d*-fenfluramine, and this depression of motor activity was noticeable until 35 minutes after food had been returned [after 5 minutes $F(2,33) = 8.637$; $p < 0.001$; after 35 minutes $F(2,33) = 4.383$; $p < 0.05$]; by 40 minutes into the recording it was non-significant. Animals treated with sibutramine also showed less locomotor activity than controls over the first 35 minutes post food re-introduction [$F(2,33) = 4.383$; $p < 0.05$], an effect that first became significant after 15 minutes access to the feeding jar [$F(2,33) = 8.784$; $p = 0.01$]. However, after 40 minutes the effects of both drugs had subsided, and they remained no different from controls thereafter.

CT9. Drug treated animals showed no variation from controls at any point during the 60 minutes following food presentation at this circadian time.

CT13. Following food re-introduction animals treated with *d*-fenfluramine showed a brief locomotor activation compared to controls [$F(2,33) = 4.545$; $p < 0.05$ after the first 10 minutes] which had subsided by 15 minutes. Sibutramine treated animals never differed from controls over the course of the hour following access to the feeding jars.

CT17. Sibutramine treated animals never exhibited any differences from control conditions. Animals treated with *d*-fenfluramine showed a brief initial locomotor depression [$F(2,33) = 4.270$; $p < 0.05$ after 5 min] that was non-significant again by 10 minutes after food re-introduction. Total activity in *d*-fenfluramine animals was again significantly reduced after 30 minutes [$F(2,33) = 3.842$; $p < 0.05$] and cumulative beam breaks this time remained significantly reduced through to one hour after the return of food [$F(2,33) = 3.299$; $p < 0.05$].

CT21. Cumulative activity levels never differed from control animals with either drug treatment in the hour following food presentation.

Collated data. **Figure 5.5.6** summarises the total activity recorded and activity over the first 20 minutes following the re-introduction of food at different points of the circadian cycle. A two-way ANOVA showed there to be an effect of both circadian time [$F(5,198) = 6.667$; $p < 0.001$] and drug [$F(2,198) = 6.140$; $p < 0.01$] on total activity in the hour after food was returned, but no interaction over the whole hour [$F(10,198) = 1.201$; $p < 0.292$; not significant]; a significant interaction [$F(10,198) = 2.358$; $p < 0.05$] was observed over the first 20 minutes following the return of food. Across all circadian times sibutramine significantly reduced total activity compared to controls [$F(2,198) = 6.140$; $p < 0.01$] while *d*-fenfluramine did not. Across all drug treatment, locomotor activity was significantly increased at CT21 compared to CT1, CT5 and CT9 [$F(5,198) = 6.667$; $p = 0.001$, $p = 0.001$ and $p < 0.001$ respectively], and at CT17 compared to CT9 [$F(5,198) = 6.667$; $p < 0.05$].

Looking solely at the saline condition, activity was increased at CT21 versus CT9 over the 1 hour following food return [$F(5,66) = 4.031$; $p < 0.01$], whereas in the first 15 minutes post food re-introduction activity was significantly increased at CT5 compared to CT1, CT9 and CT13 [$F(5,66) = 3.520$; $p < 0.05$ in each case]. When the animals were treated with *d*-fenfluramine, locomotor activity was higher at CT13 compared to CT1, CT5, CT9 and CT17, but only over the first 5 [$F(5,66) = 5.628$; $p < 0.05$ compared to CT1 and CT5; $p < 0.01$ compared to CT9 and CT17] or 10 minutes following food return [$F(5,66) = 2.960$; $p < 0.05$ compared to CT17 and CT9]. Animals showed levels of activity that were significantly higher at CT5 than CT1

early in the post-food period [$F(5,66) = 2.912$; $p < 0.05$ at 10 minutes] after receiving sibutramine and overall activity was higher at CT17 and CT21 than CT1 – significant after 50 minutes [$F(5,66) = 2.972$; $p < 0.05$], but not so after 60 minutes in this case.

5.4 Discussion

Looking at the results, there does appear to be a differential effect of both sibutramine and *d*-fenfluramine compared to controls at different circadian times. When treated with sibutramine animals exhibited slight signs of depressed locomotor activity, but only at CT1 and CT5 – early and mid-light phase – and even then the effects were not significant over the whole period, but visible over the first half (CT5) or latter half (CT1) of the hour following the presentation of the feeding jars. When looking at collated data from all 6 circadian timepoints the decreases in total activity, which though present at each CT, bar CT9, were not significant in isolation, indicate a consistent and significant reduction in locomotor activity in animals dosed with 1.67 mg/kg sibutramine in the 60 minutes following the return of food. By contrast, *d*-fenfluramine treatment produced differences to saline injections at CT5, CT13 and CT17, but over 60 minutes of post-food recording *d*-fenfluramine did not significantly reduce activity irrespective of circadian time. As with sibutramine, the effects of *d*-fenfluramine at CT5 were not significant over the full hour, but the decreased activity was noted immediately and for 35 minutes. This compares to the effect at CT17, where the opposite was true; whereas during the mid-light phase the effect was an initial decrease not significant beyond 40 minutes, in the mid-dark phase the effect was significant from 30-60 minutes after food was returned. Though an initial depression was noted with *d*-fenfluramine at CT17 it was not a significant decrease in activity compared to controls (**figure 5.5.6**, first panel). Similarly, at CT13 *d*-fenfluramine produced an initial effect that didn't last more than 10 minutes; at the early dark phase timepoint however, this effect was an increase in locomotor activity. The animals tested at CT21 exhibited the greatest activity in the hour long post-food session, regardless of treatment, whilst the nadir for activity was CT9 or CT1 for sibutramine-treated animals. Within treatment boundaries, a look at the cumulative data indicates brief differences in activity as a result of circadian time early in the post-food recording but no extensive difference in activity levels over the full hour of recording – only when animals had been injected with saline did circadian rhythms prove a significant modifier of total activity. Even then the only significant differences were between the nadir of behavioural activation at CT9 and the zenith at CT21. Although statistical examination revealed differences between the levels of activity shown across the circadian cycle with animals given 1.67 mg/kg sibutramine,

activity levels were not significantly different at any one circadian time from any other.

To tackle the differences due to circadian rhythmicity first, it is wholly unsurprising that the total activity counts tended to be highest at points during the dark phase, and that the late light phase was the time of least activity. This fits in with rats being nocturnal creatures. The relative lack of activity at CT9 also correlates well with observations from the BSS at this time [chapter 7, below]. On the other hand, the total differences in activity levels observed across the circadian cycle in these experiments are actually quite small. Under control conditions only the peak and lowest activity level were significantly separable over 60 minutes of post-food recording, but looking at all drug conditions, the peak activity at CT21 was significantly higher when compared to all points during the light phase, and activity during the mid dark-phase was also significantly higher than at the nadir of locomotion at CT9. It is perhaps surprising that there were not more marked differences in activity levels, but this could be down to the design of the experiment.

A couple of possible procedural explanations spring to mind; firstly, the feeding jars themselves are sizeable, and occluded a number of the infra-red beams of the locomotor monitor when placed into the cage, preventing these beams being broken (again) during the course of the recording and thus discounting any movement covered solely within the confines of the occluded beams (see **figure 5.5.7**). This could be of importance during the dark phase, when one would expect not only general activity to be higher but also to see a specific increase in the feeding drive and animals are likely to spend more time at or around the food source.

Alternatively, the food deprivation of 6 hours meant that even at the mid-light phase point, CT5, the animals had been food deprived for an hour of their more active period; the resulting hunger motivation could have been a levelling factor, increasing baseline activity at CT1 and CT5 to statistical equivalence with dark phase levels.

A decrease in locomotor activity with *d*-fenfluramine is consistent with previous reports that this drug has sedative actions in the rat (Aulakh *et al.*, 1988, Callaway *et al.*, 1993, Ziance *et al.*, 1972), though here the locomotor behaviour inhibited was

home-cage behaviour as opposed to that shown on exposure to a novel environment, and the dosage used in the present work is smaller. It is notable, however, that the duration of the decrease showed circadian differences; at mid-light the drug-induced inhibition of activity wore off after 35-40 minutes, whereas in animals dosed mid-dark phase the onset of the effect was seen later and the effect remained statistically significant. The mid-light result could be explained by the marked tail-off in activity shown in controls; after the 35 minute mark, control animals were averaging 10-15 beam breaks per 5 minute time bin, compared to around 40 beam breaks per time bin under *d*-fenfluramine treatment conditions. The most likely explanation for the tail off in controls is their progression to a post-prandial resting state as would be expected if they fed to satiety. One might expect a similar progression in the *d*-fenfluramine treated animals given the apparent advance in the satiety sequence with this drug (*e.g.* Halford *et al.*, 1995, 1998; chapters 4 and 7) but observations carried out in the mid-light phase by Willner and colleagues (Montgomery and Willner, 1988; Willner *et al.*, 1990; McGuirk *et al.*, 1992*a*) report acute fenfluramine as disrupting the resting state, and the current observations are consistent with this eventuality. By contrast, when tested at mid-dark phase, when activity is naturally higher, animals under control conditions did not exhibit a tail-off in activity, nor did *d*-fenfluramine treated animals show an activity spurt 30-60 minutes after food was returned; the significant difference in activity between the treatments remained throughout an hour of recording, indicating a possible sedative effect. Rats generally show a higher baseline activity during the dark phase, and though not always significant the data presented here do suggest this. Increased general activity could be a reflection of the lack of a sustained resting period in saline treated animals because of a quick progression to the next meal at this time. Observations from the BSS experiments at CT17 would seem to support this, with feeding counts suggesting a second meal after 40-50 minutes under control conditions. The observations with *d*-fenfluramine in the BSS at CT17 parallel the findings here with a significant reduction in activity scores over the hour recording compared to controls. The pattern of observations also shows a second meal after 45-50 minutes, and the general profile [**figure 7.5.13** chapter 7] resembles a compressed version of the saline profile, possibly indicating a mild sedative effect.

There are signs that at times *d*-fenfluramine is exerting a sedative effect, but a cautionary note in interpreting these findings is that in the present experiments the

locomotor depressant effect was only observed at two timepoints where the *d*-fenfluramine treated animals were not their own controls. This is further flagged as potentially confounding given dosing with 1.0 mg/kg *d*-fenfluramine did not produce a CT-independent decrease in total activity as was seen with sibutramine; this dose of *d*-fenfluramine was also not seen to be sedative in exploratory locomotor activity experiments described in the next chapter [6, below].

The brief initial activation that was seen at CT13 is at odds with all previous reports of *d*-fenfluramine impact on locomotor activity (Aulakh *et al.*, 1988; Callaway *et al.*, 1993, Ziance *et al.*, 1972). This effect could perhaps be explained by a displacement of early feeding behaviour at a time when feeding drive is high at the beginning of the rat's active phase. Perhaps the state of partial satiety induced by the drug causes the rat to exhibit other activity rather than feeding from the newly presented food jar. This activation was only seen at CT13, and was only present after 5 and 10 minutes into the post-food recording; at this point activity levels in *d*-fenfluramine treated animals at CT13 were also significantly higher than in animals dosed with this drug at other circadian times. This finding is not backed up by a corresponding increase in activity scores compared to saline treatment over this first period from BSS experiments carried out at CT13 [chapter 7]. While activity scores from the BSS are not directly representative of locomotor activity, the discrepancy does suggest the current findings are anomalous. No sign of this locomotor activation was seen at any other circadian time in the current paradigm, nor was it observed in exploratory locomotor activity studies. Moreover, the long since reported sedative effects of (*d-*)fenfluramine (Aulakh *et al.*, 1988; Callaway *et al.*, 1993, Ziance *et al.*, 1972) tally with other findings presented here and in chapters 4 and 6 where a higher dose of *d*-fenfluramine (3.0 mg/kg *i.p.*) was used in certain cases. However, these previous reports were both at higher doses than the 1.0 mg/kg used in the above experiments and found to be independent of 5-HT release (Callaway *et al.*, 1993), leaving open the possibility that the specific neurochemical backdrop at CT13 is such that release of 5-HT from neuronal stores could be the signal for behavioural activation. If so, this would bear further investigation, as behavioural activation could lead to an increase in metabolic rate, a desirable effect in anti-obesity therapy however small it may be.

The effects seen with sibutramine are interesting, too. At five of six circadian times at which trials were carried out animals showed less activity after having been dosed with sibutramine than with saline. Although at each individual circadian time the difference in total activity over an hour of recording was not significant, when looked at independently of circadian time these results show a significant decrease in total activity compared to controls. This finding is consistent with the assertion that sibutramine enhances satiety and advances the BSS, as animals that have advanced to a state of post-prandial resting would not exhibit high levels of activity. It could also be indicative of a low-level sedative effect.

Looking at the individual circadian times, at early and mid light phase (CT1, CT5) there were signs of significant reductions in activity when animals were treated with sibutramine. At CT5 the activity chart for sibutramine treatment (**figure 5.5.5b**) plateaus off before the saline curve, and at a lower level of activity. This finding is consistent with an accelerated behavioural satiety sequence, and the idea that locomotor activity plateaus as animals satiate, approaching and reaching a state of post-prandial resting. At CT1, however, the trace shows fewer signs of the tail-off in activity, and is perhaps more indicative of a non-specific sedative effect. Comparing this to satiety sequence observations reveals that resting is increased in sibutramine treated animals as early as 10 minutes into the post-food observation at both CT1 and CT5 and although total activity scores in that paradigm were not significantly reduced, feeding did exhibit a concurrent decrease. Further comparison with BSS behaviour profiles from CT1 and CT5 also suggest that the effect at CT5 is due to satiety-induced resting, whilst at CT1 there was an initial active response giving way to resting as opposed to a robust feeding observation and progression through a satiety sequence. Previous findings that sibutramine – even at higher doses – does not affect locomotor activity, positively or negatively, despite raising extracellular dopamine levels in the nucleus accumbens (Rowley *et al.*, 2000) would argue against the decrease in locomotor activity seen with sibutramine in this paradigm at CT1 and CT5 being a sedative effect at the 1.67 mg/kg dose used. Experiments looking at exploratory locomotor activity were also carried out as a follow up to the data presented here to investigate whether acute sibutramine could be exerting a sedative action [chapter 6] but while in one experiment a higher dose of 5.0 mg/kg exhibited sedative activity, this finding could not be reproduced in subsequent experiments. Not

did 1.67mg/kg of sibutramine show significant signs of sedation or other decrease in locomotor function.

Finally, the BSS experiments described in chapter 7 (below) revealed that while animals dosed with sibutramine were observed resting approximately 30% more often than when given saline during testing at CT1 and CT5, the difference in total resting observations was much greater during the dark phase when sibutramine also led to increased total resting observations relative to controls, yet locomotor activity at these circadian times was not significantly decreased by sibutramine when the circadian times were examined independently.

All this seems to paint a mixed picture as to the effects elicited by both drugs in this locomotor paradigm, but it appears that sibutramine, at a dose of 1.67 mg/kg, does tend to reduce locomotor activity. The reduction is slight, however, requiring a large sample size (resulting from pooling data across the circadian cycle) to show as statistically significant. This reduction in locomotor activity does not appear to be a direct sedative effect and it is more noticeable at times of low activity, not specifically observed at times of peak activity. Furthermore this dose of sibutramine proved to have no impact on exploratory locomotor activity as described in chapter 6, below. The reduced locomotor response is therefore more likely to be due to the satiety enhancing effect of sibutramine and the resultant increase in resting and inactivity as the animals reached satiety sooner following the presentation of food when treated with sibutramine than they did when dosed with saline. This theory fits generally with both the above results and the findings from BSS studies described in chapters 4 and 7 (dealing with BSS experiments), and the existing work suggesting sibutramine acts to enhance satiety (Halford *et al.*, 1995, 1998, Heal *et al.*, 1998*a*). By contrast, *d*-fenfluramine showed no overall effect on locomotor activity, but did seem to have a locomotor-activating effect over the first 10 minutes of the post-food period at CT13. Depressant effects on locomotor activity were present at times during the tests at CT5 and CT17, and while *d*-fenfluramine is a known sedative at higher doses (Aulakh *et al.*, 1988; Callaway *et al.*, 1993, Ziance *et al.*, 1972) the 1.0 mg/kg dose used in these trials did not reduce exploratory locomotor activity [chapter 6, below]. These results should be treated with caution because these two circadian times just happen to be two where *d*-fenfluramine was tested in a separate group of animals, rather than using

them as their own control; however, the depression of activity does mirror decreases seen in activity scores from the BSS [chapter 7, below].

In short, while there are some potentially interesting significances thrown up by investigating the minutiae at each circadian time independently, the findings above suggest that *d*-fenfluramine does not, as a rule, significantly reduce locomotor activity at the dose used, while sibutramine does so in a way consistent with enhancement of satiety and early induction of post-prandial resting rather than by causing sedation. Moreover the effect of each drug does not appear to be different when administered at different circadian times, at least not over a 60 minute period – the full length of both these recordings and the BSS observations described in chapter 7.

5.5 Figures for Chapter 5

Figure 5.5.1 Photograph of one of the cages used for locomotor activity studies (both the exploratory and circadian experiments) shown *in situ.*

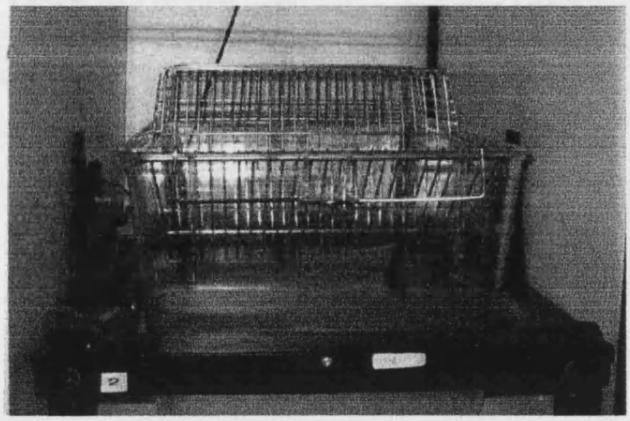


Figure 5.5.2 Photograph showing the locomotor monitor *in situ* without the cage.

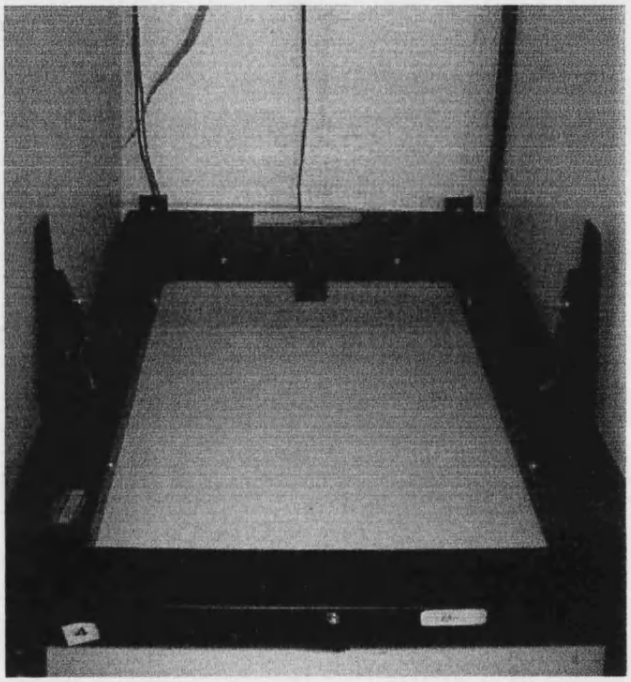


Figure 5.5.3 Photograph illustrating the layout of the 7 infra-red cross-beams arrayed in the locomotor monitor. Obstruction of a beam would generate a count.

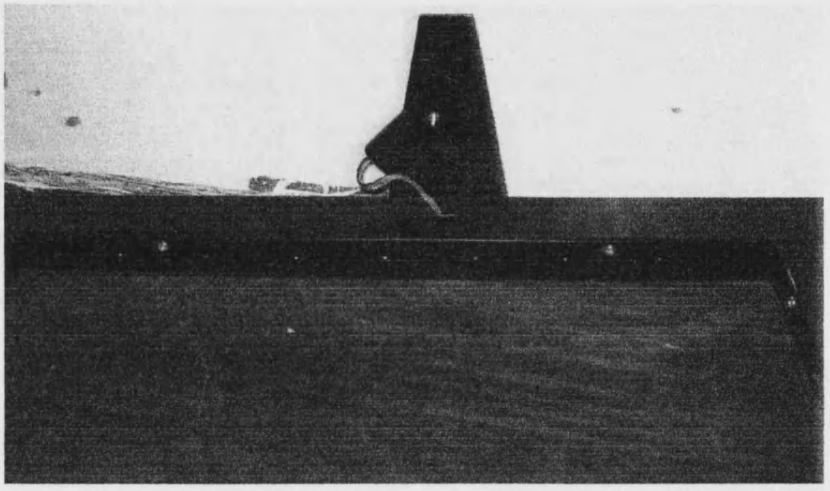


Figure 5.5.4 Photograph illustrating the layout of the 4 infra-red beams aligned with the length of the cage.

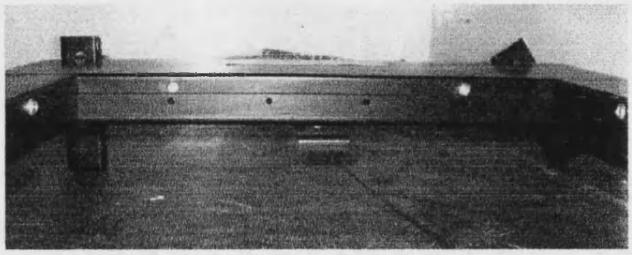
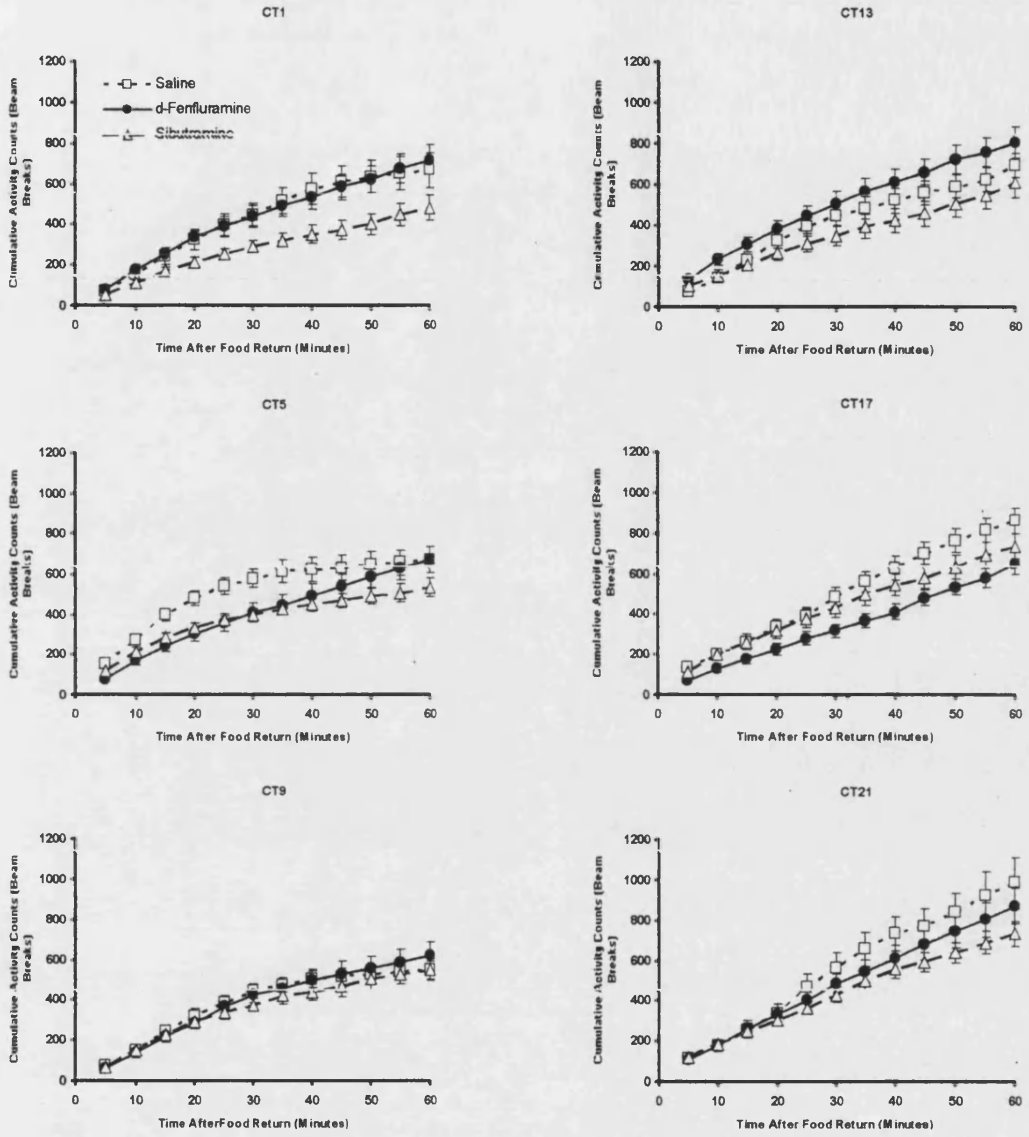


Figure 5.5.5



Cumulative mean (± standard error) beam breaks over the hour following the return of food at times spread across the circadian cycle – early, mid and late light (left column) and dark (right column) phase. *n = 12* in each case. The individual graphs follow, for clarity.

Figure 5.5.5a Locomotor activity following food presentation; recorded at CT1.

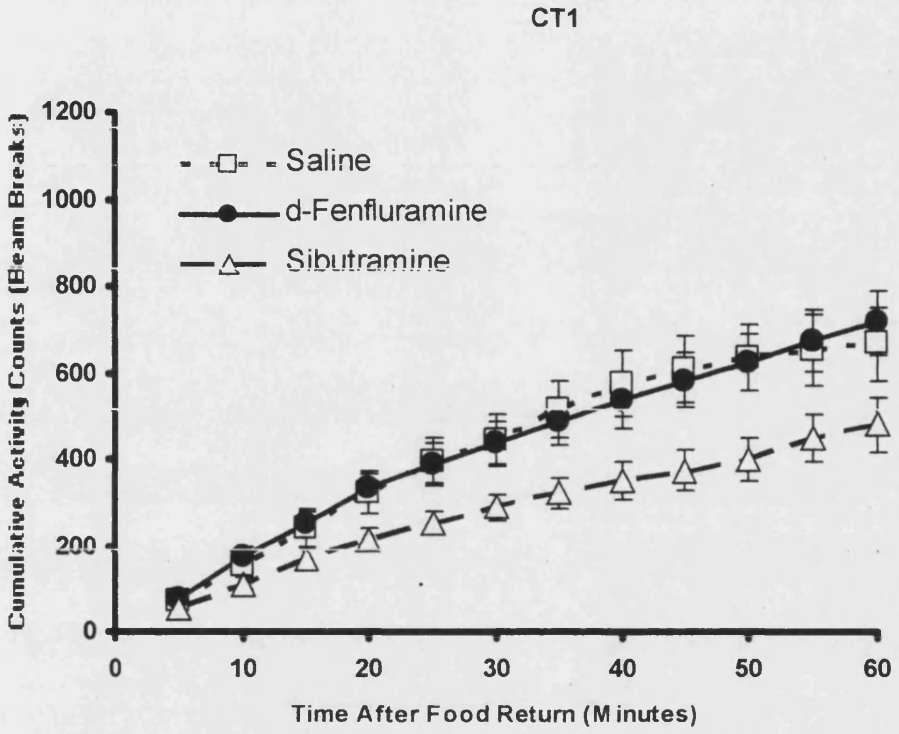


Figure 5.5.5b Locomotor activity following food presentation; recorded at CT5.

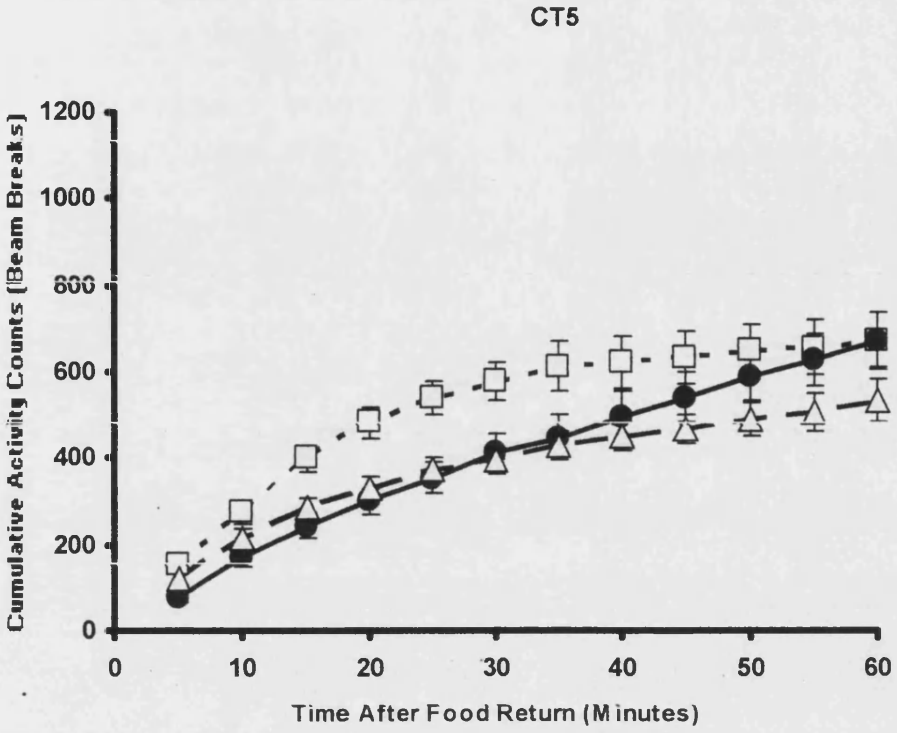


Figure 5.5.5c Locomotor activity following food presentation; recorded at CT9.

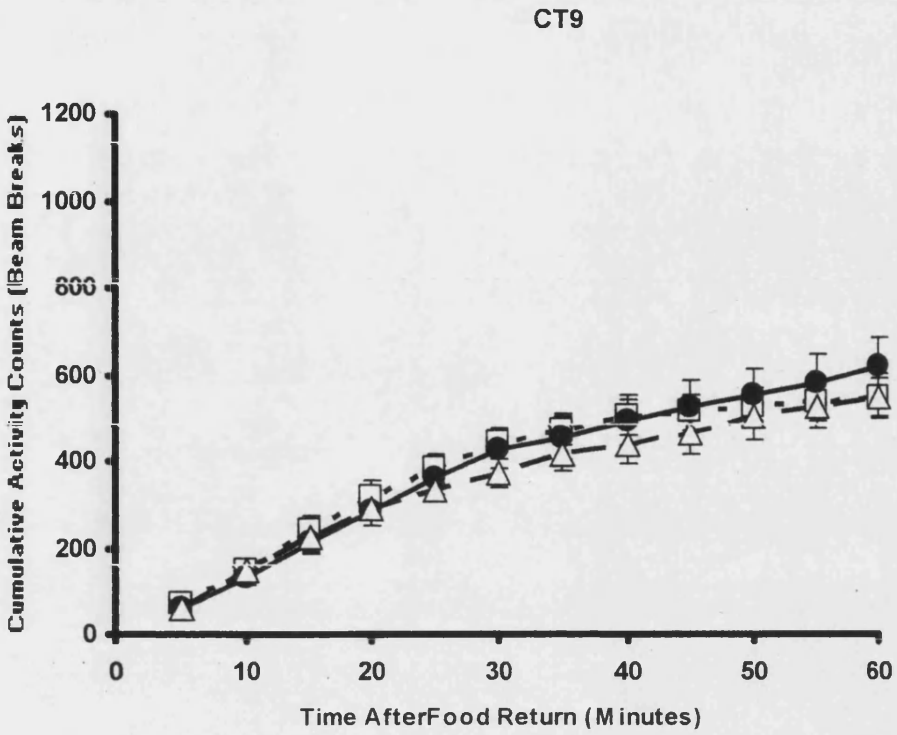


Figure 5.5.5d Locomotor activity following food presentation; recorded at CT13.

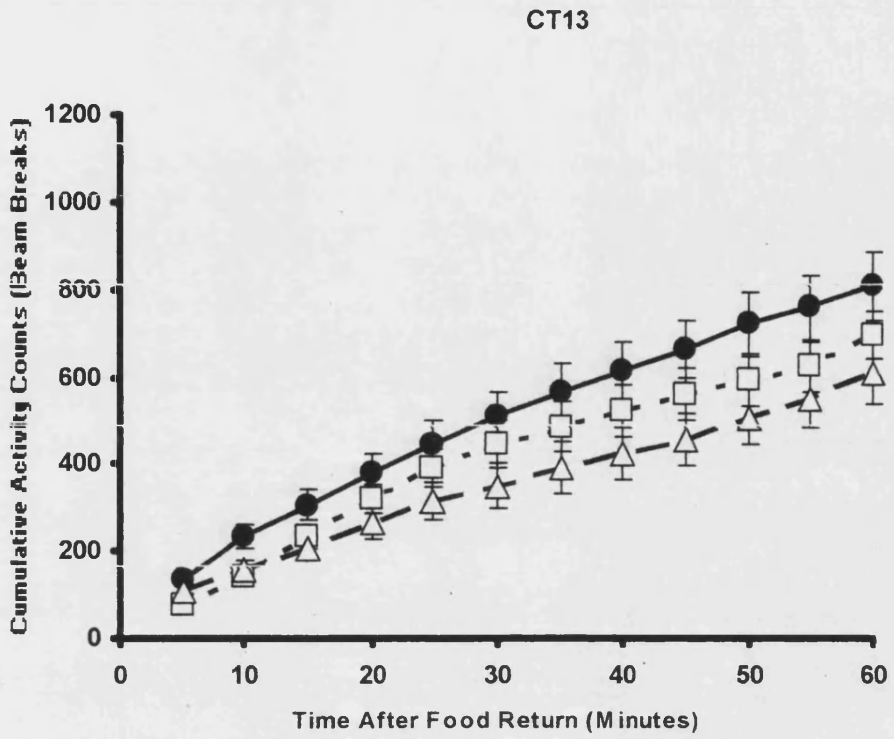


Figure 5.5.5e Locomotor activity following food presentation; recorded at CT17.

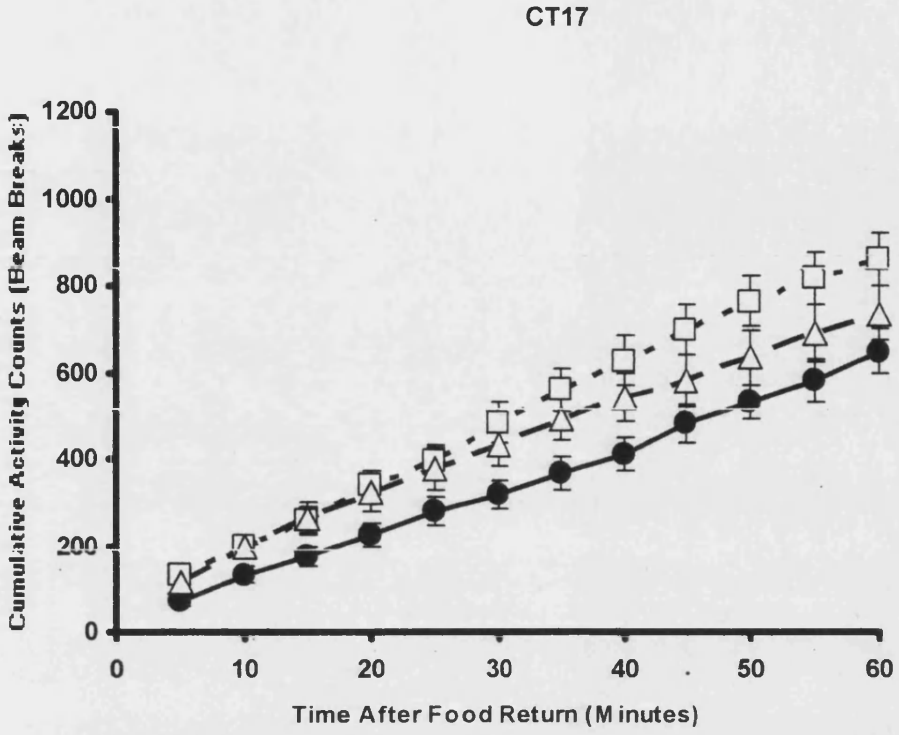


Figure 5.5.5f Locomotor activity following food presentation; recorded at CT21.

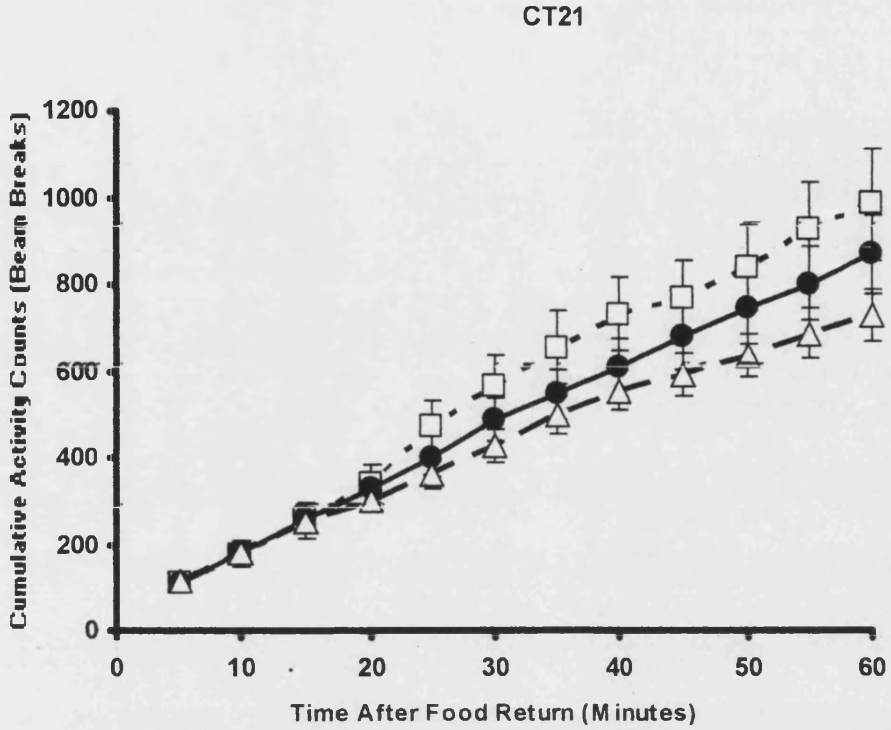
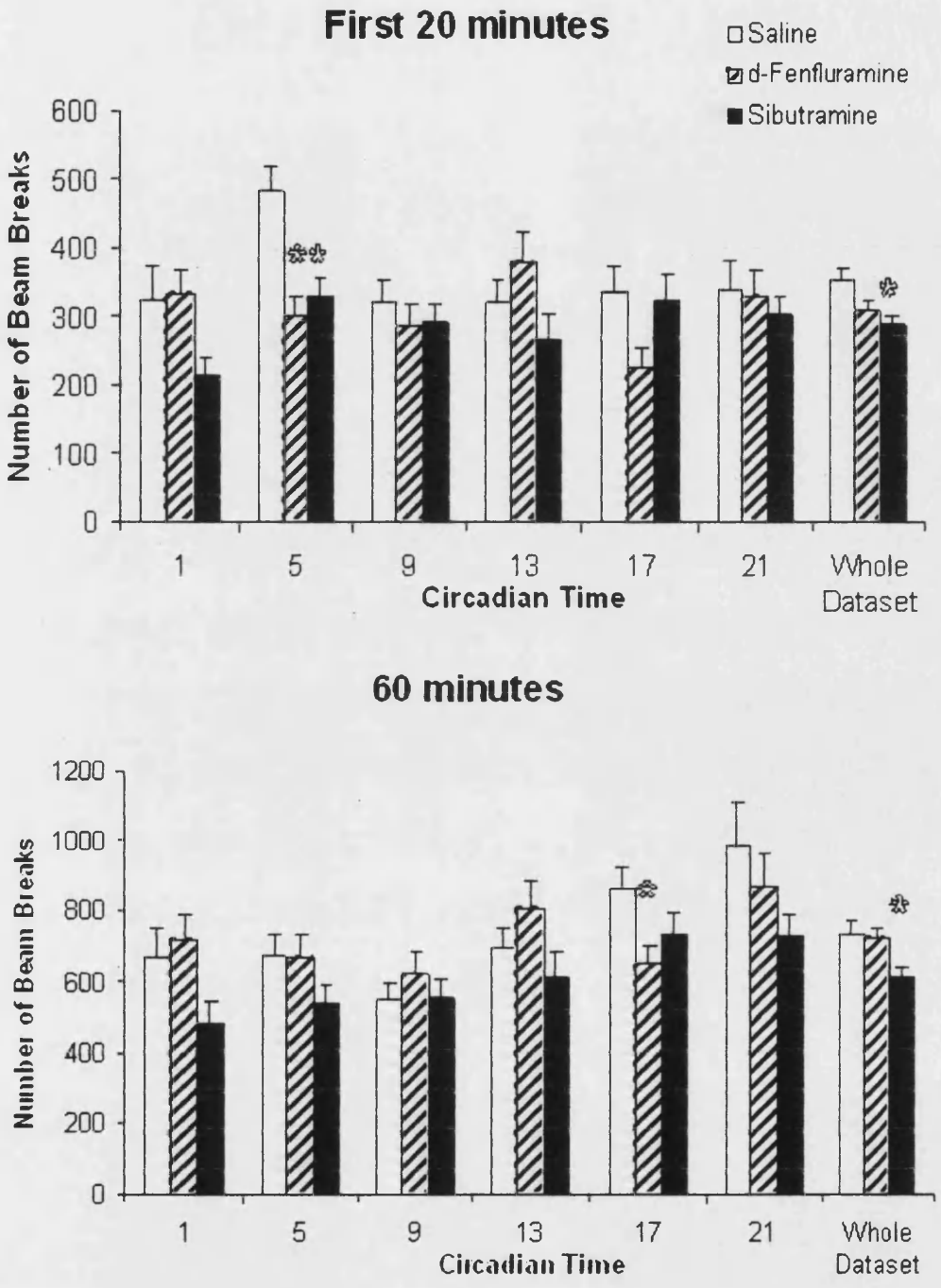
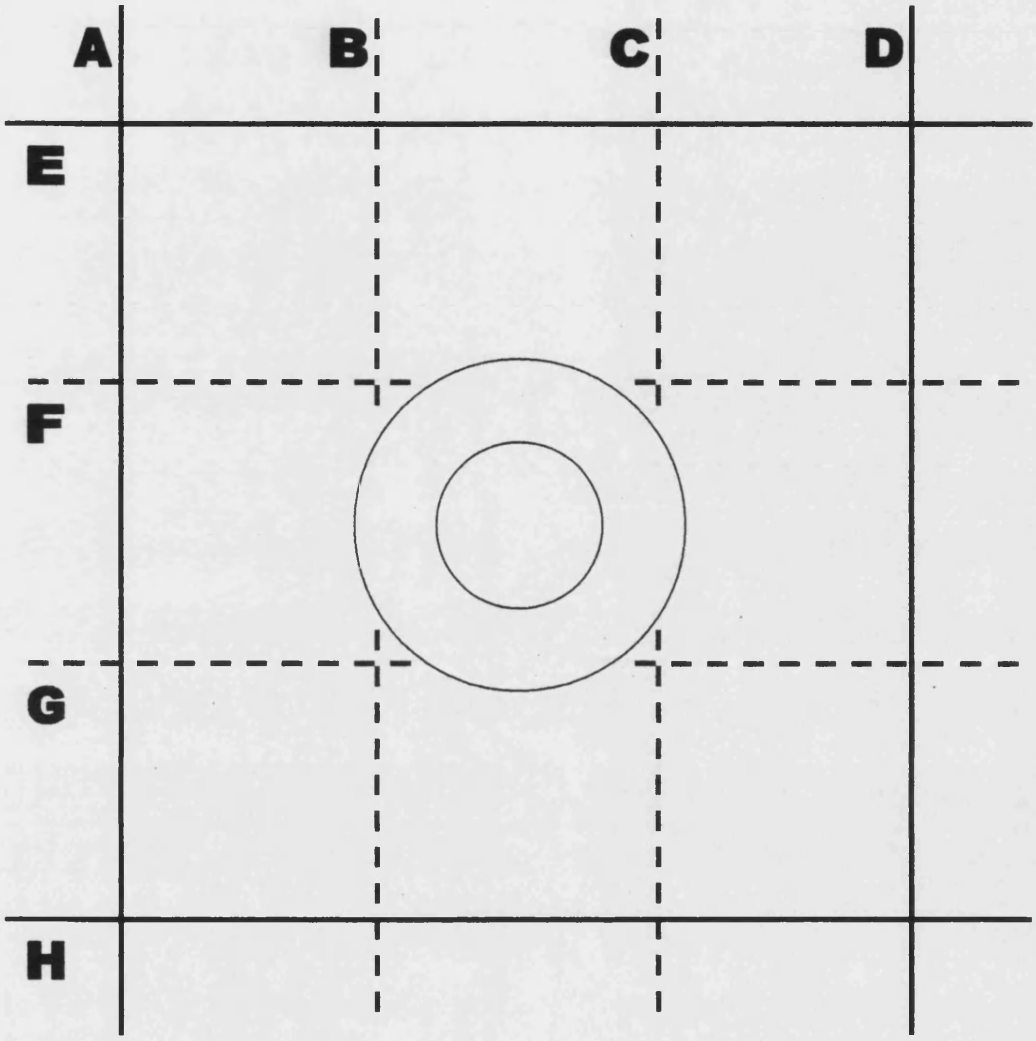


Figure 5.5.6 Variation in activity levels with sibutramine, *d*-fenfluramine or saline across the circadian cycle after 20 minutes or 60 minutes of recording.



$\clubsuit$ $p < 0.05$ compared with saline treatment at a similar circadian time.

Figure 5.5.7



Schematic illustrating how the feeding jars might produce artificially low locomotor activity counts. The highlighted beams are occluded by the jar and thus any movement of the animal within areas of the cage normally covered by these beams would not generate any activity counts. In this example, beams **B**, **C**, **F** and **G** are occluded and could not be broken by the rat; movement around the feeding jar may therefore not be recorded. Movement further from the jar would record as normal as other beams (**A**, **D**, **E** and **H**) are not occluded.

6.0 The Effect of Sibutramine and *d*-Fenfluramine on Exploratory Locomotor Activity

6.1 Introduction

There were indications from early behavioural satiety sequence observations that the high doses of both sibutramine and, more particularly, *d*-fenfluramine may have decreased locomotor activity in animals, not necessarily reflected in activity counts (the activity category being a catch-all for active behaviours not covered by other categories, including – but not limited to – locomotion). Additionally, resting tended to be increased (though not always statistically significantly) from the start in drug-treated animals. This increase in initial resting left lingering doubts about a possible sedative effect of drug treatment contributing to the decreased food intake and the alterations in the satiety sequence. Previous groups have characterised *d*-fenfluramine as sedative, with potential to decrease locomotor activity (Aulakh *et al.*, 1988; Callaway *et al.*, 1993, Ziance *et al.*, 1972), though this is not a universal finding either acutely or chronically (*e.g.* Vickers *et al.*, 2000). There are also reports that this may be caused by the drug inducing an increase in the energy cost of muscular effort (Even and Nicolaidis, 1986). Administration of *d*-fenfluramine has also been reported to disrupt the behavioural satiety sequence by decreasing resting behaviour (Montgomery and Willner, 1988; Willner *et al.*, 1990; McGuirk *et al.*, 1992*a*). This latter view is contradicted by my own experiences with *d*-fenfluramine and the satiety sequence, where I found both the onset of resting generally accelerated and the overall observations of resting increased in *d*-fenfluramine treated animals and when animals had been treated with sibutramine [chapters 4 and 7].

While the effect of both sibutramine and *d*-fenfluramine on locomotor activity were investigated in the home cage in experiments spread across the circadian cycle [chapter 5] that tied in with BSS observations described in chapter 7 (below), in order to determine the full extent of any sedative effect of either drug treatment both sibutramine and *d*-fenfluramine were examined for any effect on exploratory locomotor activity. Rats placed in a novel environment experience a drive to explore and subsequently exhibit high initial levels of locomotor activity. This increased activity is sensitive to pharmacotherapy and drugs with sedative potential will

decrease the extent to which animals explore the novel environment thus quantifiably reducing locomotor activity. Over the course of a 15 minute exposure to a novel environment, untreated animals or those given a saline control will exhibit high initial activity levels as they explore, reducing to a baseline activity level over time; characteristically, approximately 50% of their total activity is recorded in the first 5 minutes of exposure, assuming a 15 minute session. Drugs affecting exploratory locomotor activity tend to reduce the levels of activity in those first 5 minutes and quicken the drop to baseline activity levels. This test therefore is a specific examination of sedative potential and action.

6.2 Materials and Methods

Animals. Male Wistar rats (Charles River, Margate, UK; 150-250g) were used in all experiments, unless otherwise noted. Animals were kept at 21°C (+/- 1°) under a 12:12 light/dark cycle. Rats were housed in groups of eight with free access to food (CRM(E) pellets; Special Diets Services, Witham, UK) and water for 2 weeks before testing to acclimatise to the facility.

Drugs. Sibutramine hydrochloride monohydrate and *d*-fenfluramine hydrochloride (RBI, donated by RenaSci, Nottingham, UK) were both dissolved in 0.9% saline (Fresenius Kabi, Warrington, UK). Drug solutions were made up freshly each day and all injections were with 2 ml/kg of the drug solution or vehicle into the peritoneum, such that doses outlined in the individual experiments below were administered.

General Procedure. Animals were bought in from Charles River at 75-100g and housed in groups of 8 for 2 weeks to acclimatise to the new surroundings. On the day of testing, animals were weighed, dosed with drug or vehicle as appropriate and returned to their home cage. Thirty minutes later they were placed into isolation in transparent cages, to which they had not been previously exposed, which sat inside a 7x4 infra-red beam locomotor monitoring grid (see **figures 5.5.1 to 5.5.4**). Locomotor activity records were amassed for the next 15 minutes.

Statistical Analysis. Data for beam breaks in the first 5 minutes was summed and analysed by one-way ANOVA with *post-hoc* Dunnett's test. Statistics are reported with the following format: [$F(A) = B$; $p < C$] where A is the degrees of freedom and B is the F value from the relevant ANOVA; C is the probability arising from *post-hoc* comparisons.

Experiment 1. Procedure as outlined above. Animals were dosed with one of 3 doses of sibutramine hydrochloride (0.5 mg/kg, 1.67 mg/kg, and 5.0 mg/kg) or vehicle.

Experiment 2. Procedure as outlined above. Animals were dosed with one of 3 doses of *d*-fenfluramine (0.5 mg/kg, 1.0 mg/kg, and 3.0 mg/kg) or vehicle.

Experiments 3, 4 and 5. Procedure as outlined above. The doses given were a full range of sibutramine doses – 0.5, 1.0, 1.67, 2.0, 3.0, 5.0, 9.0 and 10.0 mg/kg – plus saline. Subsequent experiments were also carried out using the same basic method and dose range but varying either the dose-test interval to values between 0 minutes and 60 minutes (30 minutes in the basic procedure) or using 10% β-cyclodextrin solution instead of saline as a vehicle.

6.3 Results and Discussion

Rats placed into a novel environment exhibit exploratory locomotor activity with high initial activity as they explore the new surroundings, giving way to reduced activity the longer their activity is monitored (*e.g.* **figure 6.4.1**). **Figure 6.4.2** indicates the percentage reduction in beam breaks in the first five minutes of exposure to a novel environment in animals treated with *d*-fenfluramine or sibutramine with respect to controls. Only the highest dose tested for each drug – 5.0 mg/kg *i.p.* sibutramine and 3.0 mg/kg *i.p.* *d*-fenfluramine – significantly reduced exploratory behaviour [$F(3,28) = 10.667$; $p < 0.001$ for sibutramine and $F(3,28) = 4.243$; $p < 0.01$ for *d*-fenfluramine] in this time frame ($n = 8$ for control- and drug-treated groups in both experiments).

As far as sibutramine is concerned, this first result was at odds with previous reports that sibutramine had no effect on locomotor activity despite increasing dopamine levels *in vivo* (Rowley *et al.*, 2000). It was also contrary to findings by Brocco *et al.* (2002) that while drugs with similar mechanisms to sibutramine – selective serotonin re-uptake inhibitors (SSRIs) and serotonin-noradrenaline re-uptake inhibitors (SNRIs) – increased locomotor activity in mice exposed to novel environments, they had no effect on locomotor activity in the rat.

Attempts were therefore made to replicate this decrease and further investigate the impact of sibutramine on exploratory locomotor activity. However this finding proved to be un-reproducible despite trying experiments with varied dose-test intervals, or using β-cyclodextrin as a vehicle in addition to trying straight procedural repeats and testing a full range of sibutramine doses (*e.g.* **figure 6.4.3**). This non-reproducible nature suggests that the decrease seen after dosing with 5.0 mg/kg sibutramine in the initial experiment was an anomaly, a function of the group of animals rather than a consistent drug effect.

The decreased activity observed after treatment with the highest dose of *d*-fenfluramine is consistent with previous reports that racemic fenfluramine decreases locomotor activity (Aulakh *et al.*, 1988; Callaway *et al.*, 1993; Ziance *et al.*, 1972), as does the *d*- isomer (Ever and Nicolaidis, 1986) and both stereoisomers of the [illegible] fenfluramine metabolite norfenfluramine (Callaway *et al.*, 1993).

For both drugs, the higher two doses tested (1.0 and 3.0 mg/kg for *d*-fenfluramine; 1.67 and 5.0 mg/kg for sibutramine) showed significant impact on food intake [chapters 4 and 7] and for the longer-term satiety sequence studies the middle doses of both drugs were used. While there was a trend towards decreased locomotion in 1.0 mg/kg *d*-fenfluramine treated animals it was not statistically significant, and the results with sibutramine suggest that 1.67 mg/kg of that drug does not show sedative potential either.

To conclude, while 3.0 mg/kg *d*-fenfluramine did produce a decrease, this dose was not used for further works given the results outlined in chapter 4 (above). Neither sibutramine nor *d*-fenfluramine induced any substantial, consistent or statistically significant sign of sedation or locomotor retardation under this exploratory paradigm at doses used in the circadian studies of the BSS. This would indicate that a sedative action was unlikely to be solely or primarily responsible for the increased resting observed with both drugs in the satiety sequence. It can equally be said that the anorectic effects of both drugs do not result from displacement of feeding behaviour to increased motor activity. These findings would therefore further support the initial assertion that sibutramine and *d*-fenfluramine are drugs which advance the behavioural satiety sequence, and do so by specifically enhancing satiety.

6.4 Figures for Chapter 6

Figure 6.4.1 An example of the pattern of locomotor expression shown by rats on exposure to a novel environment.

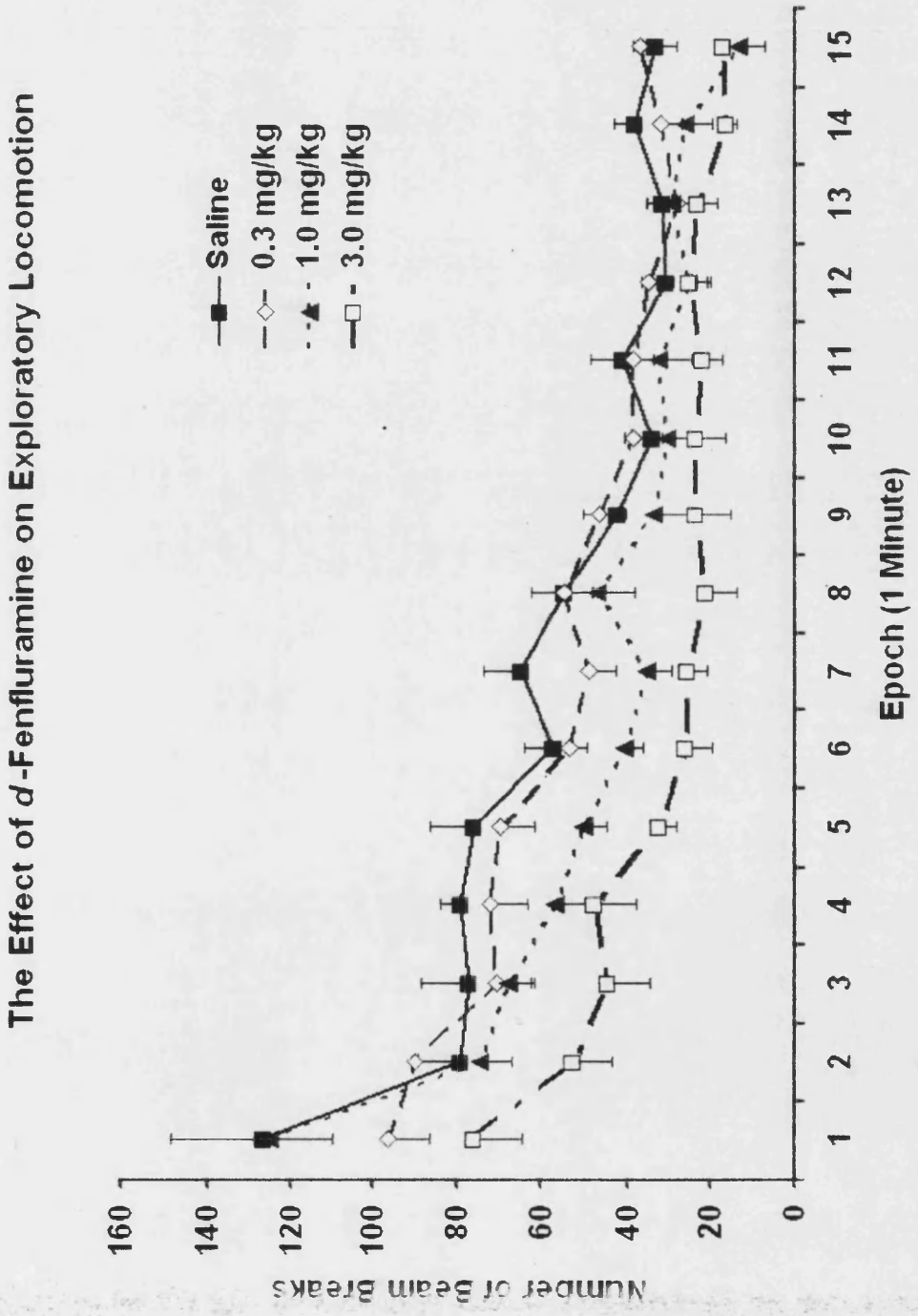


Figure 6.4.1

An example of how locomotor activity upon exposure to a novel environment decays over the duration of the recording. Animals were dosed with one of three doses of *d*-fenfluramine or saline 30 minutes prior to this recording. The aggregate number beam breaks in the first 5 epochs is roughly equivalent to that recorded over the final 10 minutes under control conditions, and drug treatment affects the expression of locomotor activity in the initial phase more than the later phase. Data are means ± standard error; $n=8$ for each group.

Figure 6.4.2 The effect of *d*-fenfluramine and sibutramine on exploratory locomotor activity.

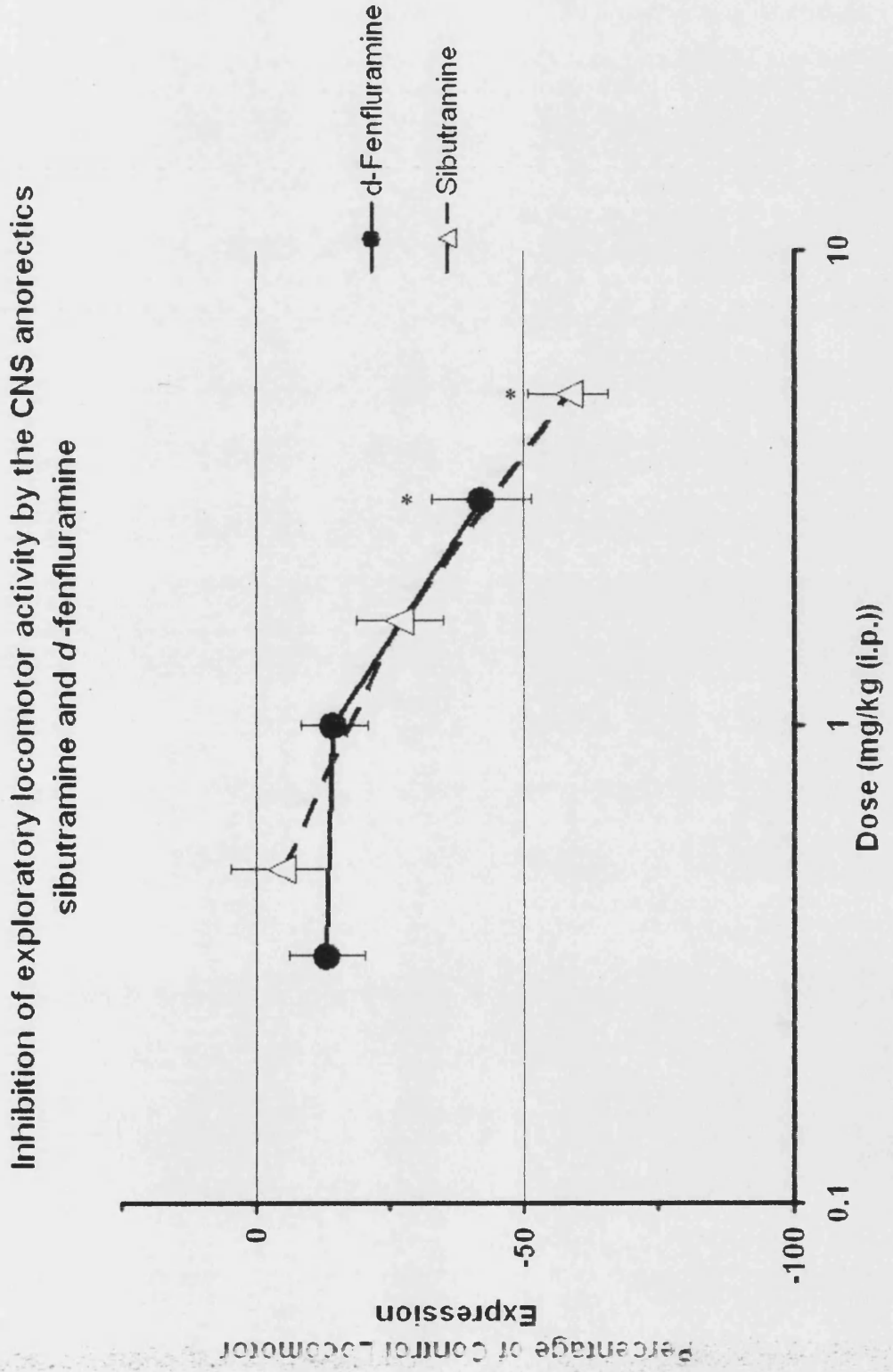
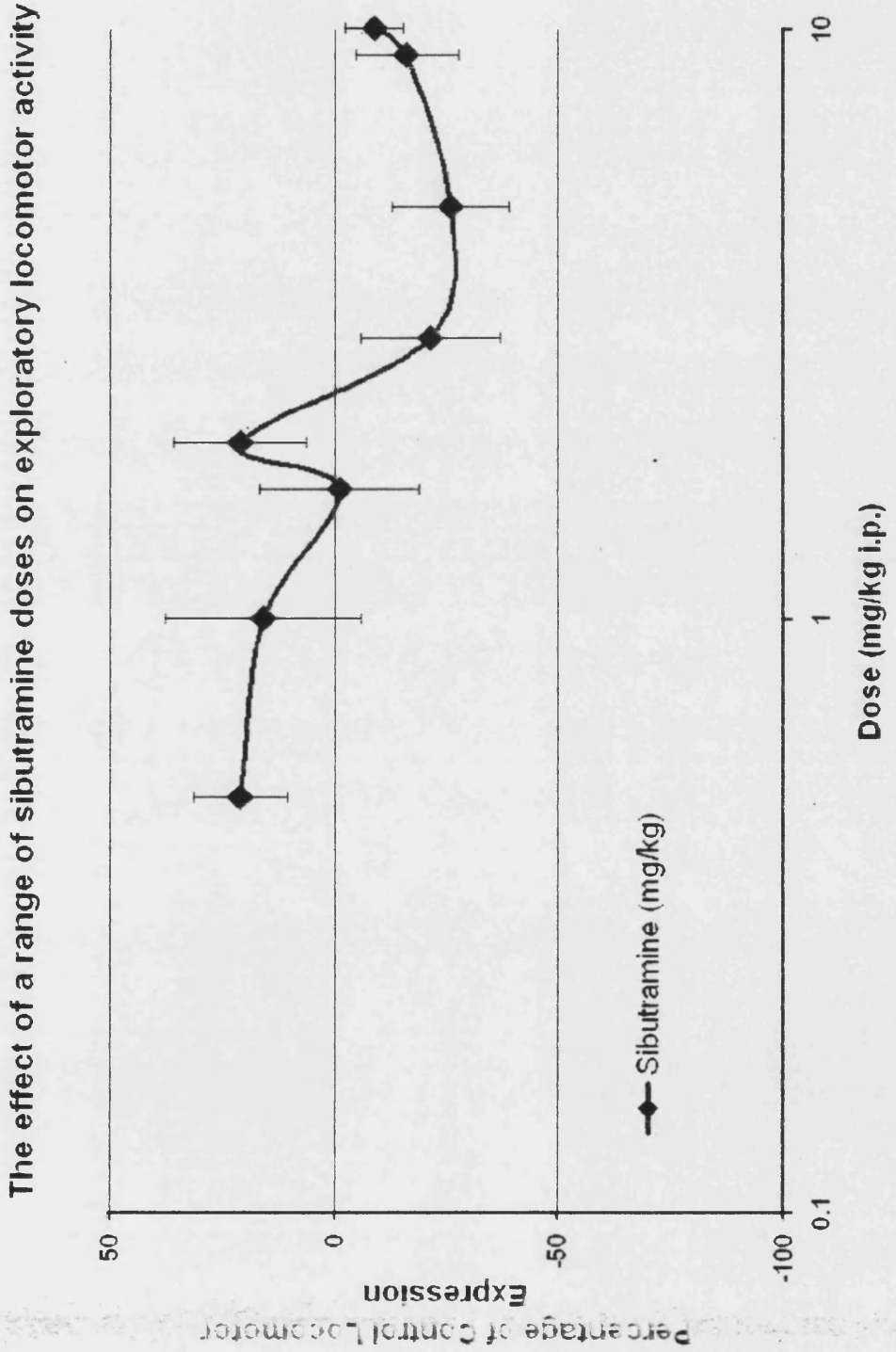


Figure 6.4.2

Percentage reduction in rat locomotor activity after administration of sibutramine or *d*-fenfluramine (each at 3 different doses) when compared to saline. Data are means ± standard error; $n = 8$ in every case; * $p < 0.001$.

Figure 6.4.3 An example of a dose-response curve for the inhibition of locomotor activity by sibutramine suggesting the significant results in **figure 6.4.2** (above) were anomalous; $n = 8$ in every case.



7.0 The Effects of Sibutramine and *d*-Fenfluramine on the BSS Measured Across the Circadian Cycle: Impact of Circadian Rhythm

7.1 Introduction

As mentioned above [chapters 3 and 4] one of the aims of the present work was to investigate the impact of circadian rhythm on the response to sibutramine and *d*-fenfluramine. As outlined in section 3.3, circadian variations affect many aspects of pharmacology; similarly feeding behaviour is under strong circadian control. This parallel makes a behavioural feeding paradigm, such as the BSS, a prime candidate for investigating interplay between circadian rhythms and drugs which affect food intake. The plethora of BSS studies to date (see Halford *et al.*, 1998, for a review, and numerous studies cited in section 4.1, above) have been carried out at a wide variety of circadian times and using an array of differing food sources and food deprivation schedules. This variation can make it difficult to draw parallels between different investigations. Observations made during the rats' natural activity phase, during which they consume the vast majority of their daily food intake, are more valuable as at this time animals tend to exhibit a high baseline of feeding behaviour and thus most rodent studies are now carried out during the dark phase. Circadian variation in activity, appetite, and pharmacological parameters can make extrapolating between observations made at different times of day difficult, and thus to fully understand a drug's action it becomes important to investigate its activity across the circadian cycle. It is with that aim that these two drugs were examined in the BSS at 6 different circadian times spread evenly over the 24 hour day.

As described above [section, 4.1], both *d*-fenfluramine and sibutramine have been examined in the BSS before now (*e.g.* Halford *et al.*, 1995, 1998) though in neither case have these drugs been investigated for effects of circadian rhythm under a consistent BSS paradigm. Racemic fenfluramine has been examined for circadian influence with respect to its effect on food intake (Davies and Wellman, 1991) and it was found to have less anorectic potency, as measured by percentage reduction in food intake, during the light phase; however, the *d*-isomer was not separately tested, nor have either the racemate or the *d*-isomer been examined for circadian variation specifically within the BSS.

7.2 Materials and Methods

Experiments 1-6. Procedures differed from the general outline described above [section 4.2] in so far as the 12:12 light/dark cycle was altered so that whilst observation was always carried at the same time (GMT) across all experiments, the subjective time at which the animals were tested differed between experiments. Over the course of the 6 experiments animals were tested at Circadian Times (CT) 1, 5, 9, 13, 17 and 21, where CT0 was lights-on and CT12 was lights-off. A different set of animals was used at each circadian time point, and in each experiment animals were dosed with saline, 1.0 mg/kg *d*-fenfluramine and 1.67 mg/kg sibutramine in a randomised treatment order. For simplicity of reporting they have been re-numbered in order of increasing circadian time.

In addition to statistical analysis carried out as described above on results from the individual experiments, data from experiments 1-6 were collated and subjected to further analysis for the effect of circadian time. The entire dataset was examined with two-way ANOVAs, using *post-hoc* Dunnett's tests for the overall effect of drug and *post-hoc* Bonferroni tests to examine the effect of circadian time, applied independently to each of the four behavioural categories. Food intake was similarly examined. Additionally one-way ANOVAs were also used to examine the effect of circadian time on each behaviour, and on food intake, within each treatment condition, with *post-hoc* Bonferroni tests where appropriate. Statistics are reported with the following format: [F(A) = B; $p < C$] where A is the degrees of freedom and B is the F value from the relevant ANOVA; C is the probability arising from *post-hoc* comparison.

With only three treatment conditions, the randomisation in each experiment was such that each treatment was administered to two animals (and thus two test cages) on every test day.

7.3 Results

7.3.1 Looking Individually at Each Circadian Time

Experiment 1. **Figure 7.5.1** shows behavioural profiles obtained from BSS observations carried out at CT1 in the early light phase. **Figure 7.5.2** illustrates the level of behaviour recorded during each discrete epoch over the course of the 60 minute observation, whilst **figure 7.5.3** shows cumulative counts for each behaviour under each drug condition. At this circadian time, 1.67 mg/kg sibutramine accelerated the offset of feeding such that cumulative feeding counts were lower than controls after 10 minutes [$F(2,27) = 7.301$; $p < 0.01$] and remained so for the hour [$F(2,27) = 19.721$; $p < 0.001$]. This dose concurrently accelerated the onset of resting, with animals observed resting more often after 10 minutes [$F(2,27) = 5.422$; $p < 0.05$], and they rested significantly more than when dosed with saline for 50 minutes of the observation period [$F(2,27) = 3.841$; $p < 0.05$]; resting was non-significantly different from saline over the hour-long observation. Activity was briefly suppressed between 20 and 25 minutes into the observation period [after 20 minutes $F(2,27) = 8.092$; $p < 0,05$; after 25 minutes $F(2,27) = 8.318$; $p < 0.05$], while grooming was unaffected. By comparison, 1.0 mg/kg of *d*-fenfluramine had an immediate inhibitory effect on feeding [after 5 minutes $F(2,27) = 3.916$; $p < 0.05$] which was preserved throughout the observation period [after 60 minutes $F(2,27) = 19.721$; $p < 0.001$], with resting immediately increased versus the saline condition [after 5 minutes $F(2,27) = 3.823$; $p < 0.05$]. This increased resting became non-significant after 50 minutes. Activity was also suppressed throughout the observation period [after 5 minutes $F(2,27) = 4.975$; $p < 0.01$; after 60 minutes $F(2,27) = 3.775$; $p < 0.05$], while grooming was increased after 10 minutes [$F(2,27) = 14.403$; $p < 0.001$], and throughout [after 60 minutes $F(2,27) = 12.618$; $p < 0.001$]. Both drugs significantly inhibited the animals' intake of food during the hour-long observation period [$F(2,27) = 18.106$; $p < 0.01$] (**figure 7.5.20**).

Experiment 2. The BSS profiles shown in **figure 7.5.4** detail the response to saline, 1.67 mg/kg sibutramine and 1.0 mg/kg *d*-fenfluramine in animals tested at CT5, mid light phase. **Figure 7.5.5** illustrates the level of behaviour recorded during each discrete epoch over the course of the 60 minute observation, whilst **figure 7.5.6** shows

cumulative counts for each behaviour under each drug condition. At this circadian time dosing with sibutramine caused an advance in the offset of feeding, with feeding counts significantly lower than when the same animals received saline between 15 minutes [$F(2,30) = 5.445$; $p < 0.05$] and 50 minutes [$F(2,30) = 4.599$; $p < 0.05$] following the return of food. Resting was concurrently increased, an effect that was noted earlier – after 10 minutes [$F(2,30) = 3.591$; $p < 0.05$] – and lasted until the same point, 50 minutes [$F(2,30) = 4.937$; $p < 0.05$] into the observation period. Sibutramine also caused a brief decrease in activity [$F(2,30) = 5.768$; $p < 0.05$] only present after 20 minutes, not at 15 or 25 minutes or any other point. This drug did not affect grooming behaviour significantly. On the other hand, *d*-fenfluramine significantly increased grooming over the full observation period [after 60 minutes $F(2,30) = 18.102$; $p \leq 0.001$], an effect that became apparent after 45 minutes [$F(2,30) = 14.422$; $p < 0.01$]. Treatment with *d*-fenfluramine suppressed activity between 20 and 50 minutes after the return of food [after 20 minutes $F(2,30) = 5.768$; $p < 0.01$; after 50 minutes [$F(2,30) = 3.913$; $p < 0.05$] compared to vehicle. Dosing with *d*-fenfluramine also accelerated the offset of feeding [$F(2,30) = 5.445$; $p < 0.05$] and the onset of resting [$F(2,30) = 9.334$; $p < 0.01$], with both effects becoming apparent 15 minutes into the observation. The effect on feeding was significant over the entire observation period [$F(2,30) = 3.614$; $p < 0.05$]; however the difference in resting ceased to be significant beyond 50 minutes. At this circadian time sibutramine did not significantly reduce food intake over the 1 hour observation, but *d*-fenfluramine did [$F(2,30) = 6.004$; $p < 0.01$].

Experiment 3. The BSS profiles shown in **figure 7.5.7** were generated at CT9, late in the light phase. **Figure 7.5.8** illustrates the level of behaviour recorded during each discrete epoch over the course of the 60 minute observation, whilst **figure 7.5.9** shows cumulative counts for each discrete behaviour under each drug condition. At this time there was little feeding drive in the animals regardless of which treatment they received – 1.67 mg/kg sibutramine, 1.0 mg/kg *d*-fenfluramine or saline. Neither drug treatment affected observations of feeding, activity or resting, nor did they produce effects on food intake. However, when dosed with *d*-fenfluramine animals were observed to groom significantly more often than after receiving saline, an effect that became significant after 10 minutes [$F(2,35) = 4.891$; $p < 0.05$] and was preserved over the course of the hour-long observation [$F(2,35) = 18.749$; $p < 0.001$].

Experiment 4. **Figure 7.5.10** presents BSS profiles garnered from observations carried out at CT13, early in the dark phase. **Figure 7.5.11** illustrates the level of behaviour recorded during each discrete epoch over the course of the 60 minute observation, whilst **figure 7.5.12** shows cumulative counts for each behaviour under each drug condition. At this time, dosing with 1.67 mg/kg sibutramine caused a noticeable decrease in feeding throughout the observation period [after 5 minutes $F(2,24) = 12.014$; $p \leq 0.001$; after 60 minutes [$F(2,24) = 15.354$; $p < 0.001$], and concurrently increased the incidence of resting observations compared to the vehicle condition after 10 minutes of observation [$F(2,24) = 6.581$; $p < 0.01$] and throughout [$F(2,24) = 8.500$; $p < 0.01$]. In comparison, 1.0 mg/kg *d*-fenfluramine prompted a similar decrease in feeding [$F(2,24) = 12.014$; $p < 0.001$] and increase in resting [$F(2,24) = 13.218$; $p < 0.05$] from the first epoch and throughout the whole period of observation [$F(2,24) = 15.354$; $p < 0.001$ and $F(2,24) = 8.500$; $p < 0.01$ for feeding and resting respectively after 60 minutes]. Neither drug showed any appreciable effect on observations of activity or grooming, but both drugs caused a significant inhibition of food intake compared to saline in the hour long experiment [$F(2,24) = 13.084$; $p < 0.001$ for *d*-fenfluramine; $p < 0.05$ for sibutramine].

Experiment 5. At the mid-dark phase timepoint, CT 17, animals generated the BSS profiles shown in **figure 7.5.13**. **Figure 7.5.14** illustrates the level of behaviour recorded during each discrete epoch over the course of the 60 minute observation, whilst **figure 7.5.15** shows cumulative counts for each behaviour under each drug condition. At this time, sibutramine (1.67 mg/kg) accelerated the offset of feeding with significantly fewer feeding observations than vehicle after 10 minutes [$F(2,33) = 4.645$; $p < 0.05$], and effect maintained throughout the observation [$F(2,33) = 9.814$; $p < 0.01$]. Resting was increased and observed earlier with this treatment; it took 25 minutes for the increase to become significant [$F(2,33) = 7.679$; $p < 0.05$] but the differential was then preserved [$F(2,33) = 13.727$; $p < 0.01$]. Animals also exhibited a brief increase in activity when dosed with sibutramine, an effect only apparent after 10 minutes of observation [$F(2,33) = 10.966$; $p < 0.05$], and gone again 15 minutes into the test period. Sibutramine decreased grooming observations with respect to control after 50 minutes [$F(2,33) = 14.878$; $p < 0.05$] and over the observation period as a whole [$F(2,33) = 12.290$; $p < 0.05$]. Treatment with *d*-fenfluramine raised total

grooming observations [$F(2,33) = 12.290$; $p < 0.05$ after 60 minutes], an effect first noticeable after 20 minutes [$F(2,33) = 6.292$; $p < 0.05$] and significant over the hour of observation, but not at all points between. This treatment also caused a suppression of activity; from 15 minutes after the food jars were presented, animals were observed as active less often when they had been treated with 1.0 mg/kg *d*-fenfluramine than when they had been dosed with saline [$F(2,33) = 7.170$; $p < 0.05$ after 15 minutes; $F(2,33) = 22.634$; $p < 0.001$ after 60 minutes]. Injections of *d*-fenfluramine also suppressed total observations of feeding [$F(2,33) = 9.814$; $p < 0.001$], noticeably lower after 10 minutes [$F(2,33) = 4.645$; $p < 0.05$], and increased total observations of resting [$F(2,33) = 13.727$; $p < 0.001$], an effect which was immediately apparent [$F(2,33) = 4.745$; $p < 0.05$]. Both drugs elicited a significant reduction in food intake compared to saline over the hour-long observation period [$F(2,33) = 11.445$; $p < 0.001$ for *d*-fenfluramine; $p < 0.05$ for sibutramine].

Experiment 6. **Figure 7.5.16** was generated from BSS observations carried out at CT21, late in the dark phase. **Figure 7.5.17** illustrates the level of behaviour recorded during each discrete epoch over the course of the 60 minute observation, whilst **figure 7.5.18** shows cumulative counts for each behaviour under each drug condition. At this time, *d*-fenfluramine (1.0 mg/kg) produced a decrease in feeding observations [$F(2,27) = 8.592$; $p \leq 0.001$] with a concurrent increase in resting [$F(2,27) = 17.930$; $p < 0.001$] over the 60 minute observation; both changes became significant with respect to the saline condition 10 minutes after food re-introduction [$F(2,27) = 5.525$; $p < 0.01$ and $F(2,27) = 7.979$; $p \leq 0.001$ for feeding and resting respectively]. Additionally, *d*-fenfluramine suppressed activity statistically significantly during the latter half of the experimental period [$F(2,27) = 4.257$; $p < 0.05$ after 30 minutes and $F(2,27) = 4.974$; $p < 0.01$ after 60 minutes with respect to saline], as well as increasing observations of grooming behaviour over the first 20 minutes [$F(2,27) = 3.917$; $p < 0.05$], but not beyond that. In comparison, sibutramine had no effect on observations of grooming or activity, but caused a similar overall reduction in feeding [$F(2,27) = 8.592$; $p < 0.01$] and increase in resting [$F(2,27) = 17.930$; $p \leq 0.001$], the differences from vehicle showing significance 15 minutes into the observation [$F(2,27) = 7.396$; $p < 0.05$ and $F(2,27) = 13.192$; $p < 0.01$ for feeding and resting respectively]. Both drugs caused a significant decrease in food intake over the hour of observation when compared to saline [$F(2,27) = 17.566$; $p < 0.001$ for *d*-fenfluramine and $p < 0.01$ for sibutramine].

7.3.2 Collated Data – Overall Effects of Treatment and Circadian Time

Effect on the BSS profile. Looking at the effect of circadian time regardless of drug treatment, feeding counts are reduced at CT9 compared to all other circadian times a difference that is statistically significant after the first epoch (CT5, 13, 17, 21, [$F(5,174) = 15.196$; $p < 0.05$ after 5 minutes; $F(5,174) = 25.528$; $p < 0.001$ after 60 minutes]) or only when looking at the full hour (CT1, [$F(5,174) = 25.528$; $p < 0.05$ after 60 min]); in all cases the differences are maintained over the hour following the return of food. Mid- and late-dark phase observations of feeding were increased relative to all light phase counts; at CT17 this effect was significant from the first epoch in every case [$F(5,174) = 15.196$; $p < 0.05$ after 5 minutes; $F(5,174) = 25.528$; $p \leq 0.001$]; the same was true at CT21, except relative to CT5 when the difference only became significant after 30 minutes [$F(5,174) = 22.401$; $p < 0.05$]. Animals were also observed feeding more often at CT17 than at CT13 [$F(5,174) = 25.528$; $p < 0.05$; over the full 60 minutes only]. Activity scores over the hour were significantly lower at CT9 than during the mid- or late-dark phase, or the early light phase [$F(5,174) = 6.066$; $p \leq 0.001$ in each case], while activity counts over the first 35 minutes of observation were significantly lower at this time than at any other point [$F(5,174) = 7.523$; $p < 0.05$]. Grooming counts were unaffected by time of observation over the hour, but animals were observed grooming more in the first 10-15 minutes at CT1 [$F(5,174) = 9.777$; $p < 0.05$, after 10 minutes] and CT9 [$F(5,174) = 7.506$; $p < 0.001$, after 15 minutes] than in the same period when tested during the dark phase. Concurrent with the decrease in feeding counts at CT9, resting counts were more frequent at this time than any other, significantly increased compared to CT1 after 25 minutes [$F(5,174) = 18.124$; $p < 0.05$] and from the first epoch when compared to all other times [$F(5,174) = 10.317$; $p < 0.05$]; in all cases the difference remained significant over the hour [$F(5,174) = 18.425$; $p \leq 0.001$]. Mid- and late-dark phase observations of resting were significantly decreased compared with counts at all light phase times. At CT 17 resting counts were significantly less than at CT1 and CT9 after 5 minutes [$F(5,174) = 10.317$; $p < 0.01$] and less than at CT5 after 40 minutes [$F(5,174) = 19.206$; $p < 0.05$]; in all cases this decrease remained significant over the full test period [$F(5,174) = 18.425$; $p < 0.01$]. A similar pattern was seen at CT21, the only difference being a slightly earlier emergence of significance in the decrease in resting compared to CT5 [$F(5,174) = 19.380$; $p < 0.05$, after 35 minutes].

Examining the overall effect of drug treatment regardless of circadian time reveals that both drugs decreased feeding observations from the first epoch [$F(2,174) = 11.973$; $p < 0.01$], an effect maintained over the full 60 minutes [$F(2,174) = 41.555$; $p < 0.001$]. Activity was observed significantly less after dosing with 1.0 mg/kg *d*-fenfluramine compared to controls [$F(2,174) = 4.288$; $p < 0.05$ after 5 minutes, $F(2,174) = 17.986$; $p < 0.001$ after 60 minutes], while grooming was observed more often [$F(2,174) = 7.363$; $p < 0.05$ after 5 minutes, $F(2,174) = 52.005$; $p < 0.001$ after 60 minutes]. Sibutramine induced a decrease in grooming compared to saline [$F(2,174) = 52.005$; $p < 0.05$ after 60 minutes], but this was only significant from 50 minutes into observation [$F(2,174) = 46.086$; $p < 0.05$]. Resting counts were increased with both drugs from the first epoch and throughout the observation period [$F(2,174) = 10.919$; $p < 0.01$ after 5 minutes, $F(2,174) = 22.594$; $p < 0.001$ after 60 minutes].

Significant interactions between drug treatment and circadian time were noted in their effects on all 4 behaviours. Although non-significant over the full 60 minutes [$F(10,174) = 1.774$; non-significant], circadian time significantly changed drug effects on feeding over the first 20-50 [$F(10,174) = 1.988$; $p < 0.05$ after 20 minutes; $F(10,174) = 1.924$; $p < 0.05$ after 50 minutes] minutes but not the first 15 minutes of the observations [$F(10,174) = 1.789$; non-significant after 15 minutes]. Resting behaviour was differentially affected by drug treatment across the circadian cycle after 15 minutes [$F(10,174) = 1.920$; $p < 0.05$] and over the 60 minute test period [$F(10,174) = 3.045$; $p < 0.01$]. A significant interaction between circadian time and drug treatment on the incidence of grooming behaviour was noted over the 60 minute observation period [$F(10,174) = 1.916$; $p < 0.05$], whilst their effects on activity showed an interaction *only* if examined over the first 35 minutes of the test period [$F(10,174) = 1.938$; $p < 0.05$] – not the first 30, not the first 40, nor the whole experiment.

Looking at the differential effects of circadian time on each distinct drug condition, feeding was observed less often under control conditions at CT9 than at any other time except CT1, differing from dark phase timepoints after 5 minutes [$F(5,58) = 5.641$; $p < 0.05$] and from CT5 after 10 minutes [$F(5,58) = 10.425$; $p < 0.05$], and

preserved throughout [$F(5,58) = 14.570$; $p < 0.01$]. Feeding was observed substantially more in the mid- to late dark phase with observations at CT21 [$F(5,58) = 14.570$; $p < 0.05$ after 60 minutes; CT21 first differed from CT1 after 15 minutes and from CT5 after 50 minutes] and CT17 [$F(5,58) = 14.570$; $p < 0.05$ after 60 minutes; CT17 first differed from CT1 after 50 minutes and differed from CT5 only over the 60 minute observation] more frequent than at any point in the light phase. Statistically significant changes in activity counts due to circadian time are seen after 15 minutes of observation; by this time saline-treated animals were observed as active less often at CT9 than at CT5 [$F(5,58) = 3.018$; $p < 0.05$]. By 20 minutes into the observations, saline treated animals were less active at CT9 than at any other time except CT13 [$F(5,58) = 5.050$; $p < 0.05$]; the decreases in activity remained significant over the hour of observation [$F(5,58) = 7.023$; $p \leq 0.001$] except differences with CT5 which became non-significant after 60 minutes. Grooming was barely affected by circadian time, although at CT9 animals were observed grooming more over the first 10 minutes than at CT21 [$F(5,58) = 3.268$; $p < 0.05$] this then ceased to be a significant difference. Resting counts were increased from the start at CT9 compared to CT5, CT13, CT17 and CT21 [$F(5,58) = 5.647$; $p < 0.05$ in each case] and after 10 minutes compared to CT1 [$F(5,58) = 8.494$; $p < 0.05$], differences that remained significant over 60 minutes [$F(5,58) = 19.291$; $p < 0.001$]. At CT5 animals were observed resting more often than at CT 17 [$F(5,58) = 19.291$; $p < 0.01$ over 60 minutes] or CT21 [$F(5,58) = 19.291$; $p < 0.001$ over 60 minutes], the differences first significant by 55 and 50 minutes respectively. After 35 minutes at CT1 the saline-treated animals were counted resting noticeably more than at CT21 [$F(5,58) = 22.605$; $p < 0.05$], and this difference was maintained thereafter [$F(5,58) = 19.291$; $p < 0.05$ over 60 minutes].

Looking just at animals when under the effects of *d*-fenfluramine, at CT1 they were observed feeding less than at CT17 [$F(5,58) = 7.084$; $p < 0.01$ over 60 minutes] and at CT9 there were fewer feeding counts than at CT17 or CT21 [$F(5,58) = 7.084$; $p < 0.01$ over 60 minutes]. All these effects were significant from the first epoch onwards [$F(5,58) = 7.552$; $p < 0.01$ after 5 minutes]. Activity counts in *d*-fenfluramine treated animals showed no variation with circadian time, but grooming counts under these conditions exhibited variation over the first 10-15 minutes of the test period. At CT1 and CT9 animals dosed with *d*-fenfluramine were observed grooming more often than at CT5 [$F(5,58) = 7.105$; $p < 0.05$, 10 minutes], CT13 [$F(5,58) = 6.205$; $p < 0.05$, 15

minutes] and CT17 [$F(5,58) = 6.205$; $p < 0.05$, 15 minutes]. Resting observations were ultimately unaffected by the circadian time of observation when looking solely at the *d*-fenfluramine condition.

Examining the sibutramine condition across the circadian cycle, feeding observations were suppressed throughout at CT1 [$F(5,58) = 5.536$; $p < 0.05$ over 60 minutes, first observed after 5 minutes] and CT9 [$F(5,58) = 5.536$; $p \leq 0.001$ over 60 minutes; first observed after 5 minutes] when compared to CT17. Feeding observations were also lower at CT9 compared to CT21 [$F(5,58) = 5.536$; $p < 0.05$ over 60 minutes; first observed after 10 minutes]. Suppression of feeding counts at CT1 relative to CT21 was also noticeable but only after 25 [$F(5,58) = 6.359$; $p < 0.05$] or 45 [$F(5,58) = 6.302$; $p < 0.05$] minutes of the observation period, and the effect was not maintained. Initial feeding was highest at CT17 (**figure 7.5.13**). Sibutramine attenuated circadian changes in activity counts, with the only differences observed between CT9 and CT17, and then only over the first 35 minutes of observation [$F(5,58) = 2.522$; $p < 0.05$]. There was no circadian variation in grooming counts under the sibutramine condition. Resting counts were significantly increased from the start at CT9 when compared to CT17 [$F(5,58) = 3.728$; $p < 0.01$ after 5 minutes] or CT21 [$F(5,58) = 5.541$; $p < 0.05$ after 10 minutes] and these differences were maintained for the whole hour [$F(5,58) = 4.649$; $p < 0.01$]. At CT1 resting observations were increased compared to CT17 [$F(5,58) = 5.966$; $p < 0.05$, after 30 minutes] and CT21 [$F(5,58) = 6.098$; $p < 0.05$, after 25 minutes] over the first half of the observation period, but not over the observation as a whole.

An illustration of the level of expression for each of the four behaviours over 1 hour of BSS observations at each circadian time and with each drug is shown in figure **7.5.19,** whilst **Table 7.5.1** summarises when drug-induced changes in observed behaviours became significantly different from saline at the various circadian times, and when looking across all circadian times.

Food Intake. Looking at the effect of drug treatments regardless of circadian time, both 1.0 mg/kg *d*-fenfluramine and 1.67 mg/kg sibutramine [$F(2,174) = 54.386$; $p < 0.001$ for both] inhibited food intake over the period of observation when compared to saline. Examining the effect of circadian time, regardless of treatment, food intake

was highest in the dark phase – intake at CT17 and CT21 was greater than intake at all light phase points [$F(5,174) = 26.832$; $p < 0.001$]; intake at CT13 was higher than intake at CT1 and CT9 [$F(5,174) = 26.832$; $p < 0.05$] – and lowest in the late light phase – food intake at CT9 was significantly reduced compared to all other time points [$F(5,174) = 26.832$; $p \leq 0.001$]). There was a significant interaction between circadian time and drug treatment [$F(10,174) = 2.801$; $p < 0.01$].

Looking at circadian variation in food intake on a treatment-by-treatment basis reveals that intake was significantly lower at CT9 than all other times in control conditions [$F(5,58) = 16.932$; $p < 0.01$], but only late dark phase intake was significantly increased compared to other times; it was significantly higher than any point in the light phase [$F(5,58) = 16.932$; $p < 0.01$ for CT1 compared with CT1, CT5 or CT9]. A differential effect is seen with *d*-fenfluramine, where the only significant difference in food intake is between peak consumption at CT17 and the nadir of intake at CT9 [$F(5,58) = 16.932$; $p \leq 0.001$]. Sibutramine treated animals simply consumed significantly less at CT9 than at any point in the dark phase [$F(5,58) = 16.392$; $p < 0.01$]. **Figure 7.5.20** illustrates the food intake at each circadian time on a treatment by treatment basis, and as a percentage of control consumption, as well as figures for the whole dataset, regardless of circadian time.

7.4 Discussion

Both *d*-fenfluramine and sibutramine have previously been described as causing an advance of the BSS (*e.g.* Halford *et al.*, 1995, 1998) and these reports are by and large in agreement with findings in this study, and the preliminary investigations described in chapter 4. The work presented here was undertaken to investigate whether the circadian time at which BSS observations are carried out has an impact on the efficacy of the drugs used, or indeed on the practicalities of the paradigm. The interactions reported above (section 7.3) suggest that the time at which the drugs are administered does indeed have a significant effect on the response to the drug – both in terms of the behavioural response (the BSS) and in terms of their effect on food intake.

Looking at the profiles in **figures 7.5.1-7.5.18**, and at the effect of the drugs on observations of feeding and resting in particular, the BSS is advanced by both drugs at almost all circadian times. The obvious exception is CT9, late in the light phase. At this time animals were largely inactive regardless of the treatment they were given, with an average of 5 of every 10 observations recorded as resting in the earliest epoch and throughout the test period, even in control conditions approximately 75% of observations were of resting behaviour **(figures 7.5.7-7.5.9)**. With no feeding response even under control conditions there was no expression of satiety, and no BSS for the drug treatments to advance. This finding is not unexpected; rats are nocturnal creatures and consume the vast majority of their food during the hours of darkness. The six hour food deprivation before the CT9 observations began encompassed only light-phase hours, and is therefore unlikely to have caused the emergence of a significant hunger drive. This ineffective food deprivation was not enough to stimulate a feeding drive strong enough to rouse the animals from their natural inactivity, and so there was no initial feeding response when presented with the food and no development or expression of the satiety sequence.

At other times both sibutramine and *d*-fenfluramine brought forward both the offset of feeding and the onset of resting to some degree or other, advancing the BSS and reducing food consumption over the hour long test period in a manner largely consistent with the enhancement of satiety. These effects consistently reached the

level of statistical significance. This is supported by the dataset as a whole. Leaving aside the circadian time of observation as a factor, both drugs accelerated the offset of feeding and the onset of resting, with the number of observations of these behaviours significantly different compared to when the same animals were given saline from the first epoch, and remaining so throughout. The inference from this is that providing there is a significant baseline feeding response then sibutramine and *d*-fenfluramine, at 1.67 and 1.0 mg/kg *i.p.* respectively, will not only reduce food intake but will do so by advancing the BSS and enhancing satiety regardless of the time and phase at which they are examined.

So does the time at which BSS observations are made matter? On the surface and from the statistics at each discrete circadian time alone it would appear not, beyond avoiding times where there is little or no feeding response. However there are both differences between the circadian times as to when the changes induced by drug treatment became significant (see **table 7.5.1**) and differing responses to the effects of circadian time between drug treatments. **Figure 7.5.19** illustrates the differences in total observations over the hour for each of the four behaviours at different circadian times. Looking at the totals for each drug across the circadian cycle like this highlights the circadian differences in behavioural expression under control conditions, but also clearly shows that the degree of circadian variation is reduced when the animals received sibutramine or *d*-fenfluramine. In the case of *d*-fenfluramine there is also a robust inhibition of activity at certain times as well as a sustained drug-induced increase in grooming, both of which are relevant to the BSS. The other factor to consider is whether the animals were exhibiting the BSS at all at any given circadian time.

Looking at some of the discrete experiments individually then, at CT1 (see **figure 7.5.1-7.5.3**) there is little evidence of satiation in controls, with the animals showing a sustained level of feeding throughout the test period. Similarly, the behavioural patterns with either drug show a lower level of feeding than controls, but one that is maintained consistently throughout the observation period (*e.g.* **figure 7.5.2**). At this time the inhibition of food intake by either drug was as strong as at any other (see figure 7.5.20), and the animals were certainly eating less and resting more after being dosed with either drug than they were after being given saline, but were they satiating

sooner or is the difference due to other factors? At this time – 1 hour into the light phase – the animals were observed resting significantly more over the course of the hour after saline dosing than were their counterparts observed at CT21 (*e.g.* **figures 7.5.16-7.5.18**), with the opposite effect being noted on feeding counts. This in itself is no surprise; CT21 in the late dark phase is a time of peak activity, while early in the light phase one would expect nocturnal animals to be less alert and less active, although activity counts did not show any change between these circadian times. After receiving a drug injection, rather than saline, these differences due to circadian time are muted (**figure 7.5.19**); in sibutramine treated animals the level of resting at CT21 is no different to that at CT1 over the full hour, though it is lower earlier in the observation, and at CT1 and CT21 animals showed no differences between observed resting levels after receiving *d*-fenfluramine. Similarly the decreased feeding at CT1 relative to CT21 seen after saline dosing was not seen after drug dosing. Again, although after receiving sibutramine the animals were observed feeding less at CT1 than at CT21 earlier on in the test period, the differences in the number of feeding observations were non-significant over the full hour. This speaks more for the efficacy of both drugs in enhancing satiety when given late in the dark phase, when the behavioural profiles (see **figure 7.5.16-7.5.18**) indicate a clearer - though longer-developing - shift from feeding to resting behaviour, than in argument against them doing so early in the light phase where this pattern is not clearly seen. It is tough to argue that an advance of the satiety sequence is taking place when the behavioural expression of that sequence is not observed. The question is thus either why the BSS is not shown at CT1, or why was it not picked up? Could this be a result of the limitations of time sampling as used here? It is always a possibility that a sampling method will produce inaccurate representations of behavioural expression but the clear evidence of a satiety sequence shown in the profiles obtained at, for example, CT5 or CT13 (**figures 7.5.4** and **7.5.13**) plus the findings described above [chapter 4] indicates that the method is solid enough to observe the BSS, so something else is likely to be behind this apparent lack of a sequence. The most obvious answer is natural inactivity, sedation or "tiredness," a consequence of the time at which the experiment was done, affecting the way in which the animals reacted to food (after having been deprived during a peak feeding time) with some animals heading straight for the food source when it was presented and others taking longer to react. Looking at the records for individual animals, this does appear to be the case.

What of the effect this may have on drug treatment, or the interpretation of the results? At CT1 the profiles (**figure 7.5.1**) and epoch-by-epoch counts (**figure 7.5.2**) suggest a blanket increase in general resting and a decrease in feeding and activity early in the test period following drug treatment, a finding backed up by the statistics (**table 7.5.1**), but the impression this leaves is more one of sedation than satiety enhancement. It is possible therefore that at a time when activity is naturally suppressed that either this inactivity masks satiation – whether natural or drug-induced – or the mechanisms of the drugs change somewhat and elicit some degree of sedative effect. Certainly this is a concern with *d*-fenfluramine, which has previously been characterised as sedative (Aulakh *et al.*, 1988; Callaway *et al.*, 1993, Ziance *et al.*, 1972), despite the dose used here being lower than that shown to decrease activity. In either case, the present results suggest that early in the light phase is not an ideal time at which to carry out BSS observations.

It is somewhat surprising then, given the situation described above at CT1, that observations carried out at CT5 produced possibly the most classical evidence of a shift in the BSS (**figures 7.5.4-7.5.6**), with a clear progression from initial feeding to a resting state observed after saline dosing. Drug treatment significantly modified behaviour from 15 minutes in, with feeding reduced and resting increased, and although *d*-fenfluramine also caused a suppression of activity observations both drugs preserved the structure of the BSS. As at CT1, both drugs also attenuated the effect of circadian time on behavioural expression as the differences in the levels of feeding and resting between mid-light and mid- and late-dark phase observations after saline dosing were not seen when drug conditions were examined. As before, this supports the efficacy of the drugs at influencing the BSS during the dark phase; the pattern of both drugs obscuring the changes caused by time of day when the animals were given saline does suggest that their efficacy is tied to when they are administered. If the drugs were acting with equal potency and efficacy across the circadian cycle, then one might expect that circadian alterations in the behavioural profiles would be the same in animals dosed identically – whether with saline or either drug. This is clearly not the case in this study and while certain changes in the BSS due to circadian time are seen after treatment with sibutramine or *d*-fenfluramine these variations are lessened compared to those seen after saline administration. Furthermore, the circadian

differences in behaviour that are maintained within the drug treatments are largely those between the nadir of behavioural expression at CT9 and the times of peak behavioural expression in the mid- and late dark phase. This is not to suggest that drug action was maintained at CT9 and not at other times; as already stated there was little baseline feeding response at this time and there were no drug-induced changes in behavioural expression other than a specific increase in grooming induced by *d*-fenfluramine. Instead it suggests the inactivity at CT9 is profound and a very dramatic drug effect on behaviours at other times would be required to reach behavioural equivalence. It is interesting, then, that such a negation of circadian effect was seen with *d*-fenfluramine with respect to observations of both activity and resting. Another cause for thought with regards to this drug is that it is clear that dosing with *d*-fenfluramine was responsible for a decrease in activity at almost all circadian times, an effect not seen with sibutramine (which had little impact on observations of activity, and no significance over the whole observation period). This raises a slight query about the nature of the drug-induced effects. Both sibutramine and *d*-fenfluramine are described as satiety enhancing drugs in previous work with the BSS (*e.g.* Halford *et al.*, 1995, 1998), and on balance both appear to exhibit satiety enhancement in the present experiments, to a roughly equivalent degree – which is expected since both doses are close to the respective ED_{50} values for 2-hour food intake (Halford *et al.*, 1995) and showed equipotent reduction of food intake in preliminary experiments [chapter 4]. Yet the effects of *d*-fenfluramine are strongly associated with a decrease in activity scores while those of sibutramine are not. The activity category was a catch-all for behaviours not associated with feeding, grooming or resting and thus a decrease in activity observations is not directly analogous to an inhibitory action on locomotor behaviour or sedation. However, the fact that the decrease observed is specific to *d*-fenfluramine, which has been previously categorised as sedative (Aulakh *et al.*, 1988; Callaway *et al.*, 1993, Ziance *et al.*, 1972) and as increasing the energy cost of muscular effort (Even and Nicolaidis, 1986), does give pause for thought. It therefore follows that some degree of sedation may play a part in mediating the effects of *d*-fenfluramine on the BSS and on food intake, although, if this is the case, the sedation is mild enough not to disrupt the BSS. A degree of sedation may be one underlying reason why there have been conflicting reports of the effect of acute (*d*-)fenfluramine on the BSS in the past: a mild sedative effect could cause an increase in activity counts later in an observation as it wears off

(Halford *et al.*, 1998) which might explain the disruption of resting seen by Willner and colleagues (Montgomery and Willner, 1988; Willner *et al.*, 1990; McGuirk *et al.*, 1992*a*). Another curiosity about fenfluramine is that here it appeared to cause a specific increase in grooming, with the largest increase observed at CT9. There is no clearly discernable reason for this change, which saw the active grooming behaviour replace passive resting seen in controls rather than active or feeding behaviours (though the reduction in resting was non-significant). **Table 7.5.1** and **figure 7.5.19*b*** demonstrate where this promotion of grooming was noticeable. Given that *d*-fenfluramine was observed to have the effect of promoting grooming at many different circadian times, albeit to different degrees, it is likely to be a conserved and specific effect of the drug but the mechanism by which the effect is mediated is uncertain. Grooming was significantly reduced by sibutramine over the hour of observation at CT17 (and when looking at data pooled from all timepoints) but at no other point. In this case it is likely the decrease in grooming is symptomatic of the general decrease in active behaviour that accompanied the drug-induced facilitation of resting behaviour.

Parallel experiments monitoring the effect of both *d*-fenfluramine and sibutramine on locomotor activity across the circadian cycle are described in chapter 5, and a look at their effects on exploratory locomotor activity – a paradigm investigating sedative potential – at doses relevant to this study can be found in chapter 6.

Another sign that there is a degree of circadian influence on the drug effect comes from the variation in when the behavioural changes induced by the drugs were detected at each circadian time (see **table 7.5.1**), although these variations are small. So, given there are variations, what does this mean? When do the drugs exhibit more potency? In percentage terms of food intake (**figure 7.5.20**), sibutramine produced the greatest inhibition of consumption at CT1 while *d*-fenfluramine suppressed intake most at CT21. Both drugs decreased food consumption at all times except CT9 (and CT5 for sibutramine); *d*-fenfluramine had a somewhat more potent effect than sibutramine but within treatment bounds the degree of inhibition was roughly equivalent across all circadian times. The present findings do not therefore support those of Davies and Wellman (1991), made with racemic fenfluramine, that the potency of this compound to inhibit food intake is higher in the dark phase than in the

light. This is more likely due to the relatively low levels of consumption seen in the present work than anything else. Peak control intake at CT21 was on average just 6.7 grams (**figure 7.5.20**), and food intake was significantly lower than this during the light phase. While the drugs were potent enough to reduce food intake reliably at individual circadian times with respect to control, the degree of error recorded relative to absolute food intake was enough to prevent conclusions as to the variation of this efficacy with circadian time.

Of perhaps more relevance than the simple measurements of food intake is the attenuation of the effects of circadian rhythm by drug treatment, *i.e.* that the patterns of circadian variation in behavioural expression seen after saline treatment are not maintained in animals treated with either drug. Muting these changes is consistent in each case with decreasing feeding and increasing rest, and in terms of the circadian variations this tends to morph the dark phase behavioural patterns towards those produced in the light phase. The argument is therefore that either the drugs show more potency in the BSS during the dark phase when there is a higher level of behavioural expression, or that the potency of the drugs is maintained in the light phase but the BSS paradigm used here was not sensitive enough to pick up the same alterations in behaviour at these times. Given that, CT9 aside, the paradigm was capable of demonstrating changes induced by the drugs at times across the circadian cycle, and was powerful enough to highlight circadian changes in behaviour when comparing results obtained from the saline condition, it is unlikely to be simply an artefact of the method. Therefore the inference is that both drugs are more effective at modifying behaviour in the dark phase despite strong effects at points in the light phase when compared to saline. This goes hand in hand with the dark phase being a more appropriate choice for observing rat behaviour on the basis that it is their natural period of activity and thus more physiologically relevant and provides a strong argument for conducting future BSS work in rats under dark phase conditions.

In summary then, both sibutramine (1.67 mg/kg *i.p.*) and *d*-fenfluramine (1.0 mg/kg *i.p.*) have a profound effect on rat behaviour and food intake measured in the BSS. This effect is present at many times over the circadian cycle, but it does require a baseline level of feeding and food intake to detect. The BSS is also a suitable method for monitoring behavioural changes related to feeding and satiation caused by

circadian rhythm, and these circadian changes in behavioural expression are attenuated by treatment with either drug when compared across the circadian cycle. This suggests that both drugs are more effective when given in the mid- or late dark phase, when animals are naturally more active. Both sibutramine and *d*-fenfluramine act to enhance satiety, although it is suggested that a mild sedation, whilst not disrupting the BSS, may play a role in the action of *d*-fenfluramine.

7.5 Figures for Chapter 7

Figure 7.5.1a Behavioural profiles generated from BSS observations carried out at CT1, early in the light phase

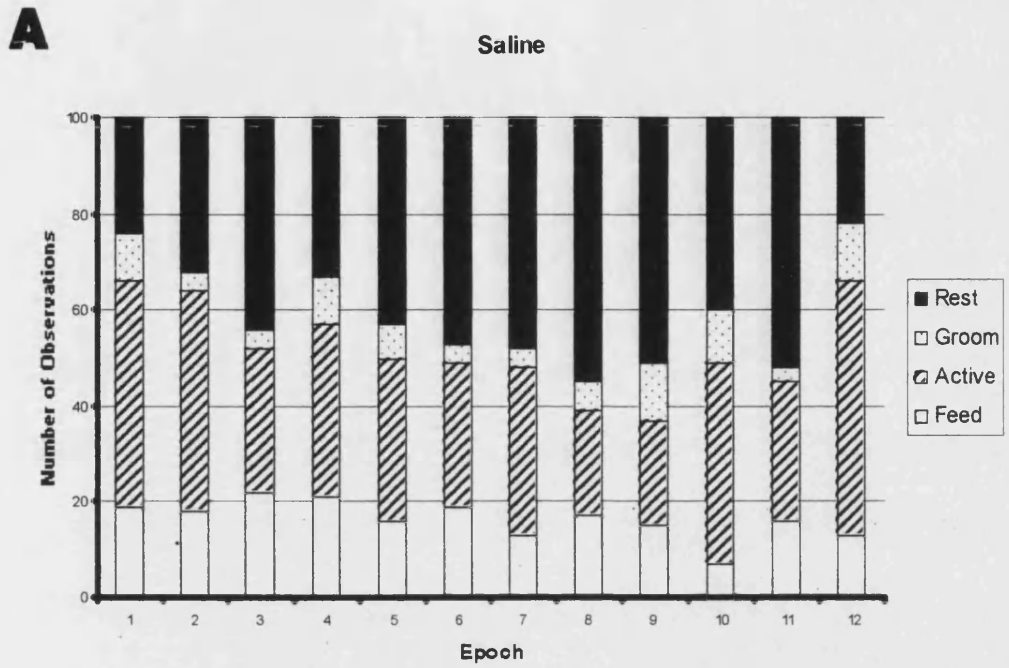


Figure 7.5.1b Behavioural profiles generated at CT1, early in the light phase.

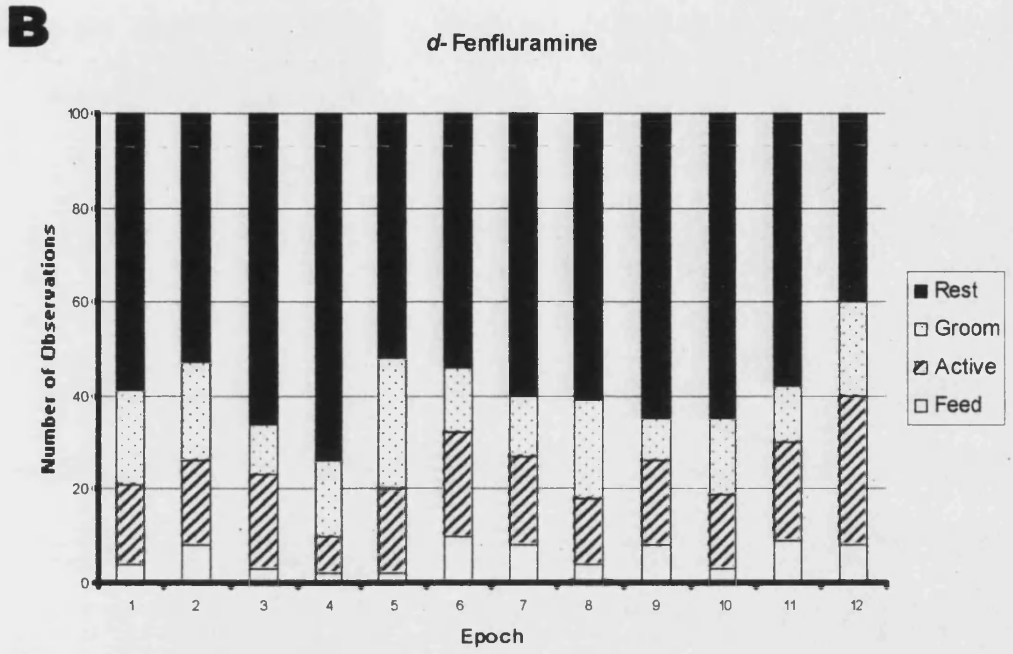


Figure 7.5.1c Behavioural profiles generated at CT1, early in the light phase.

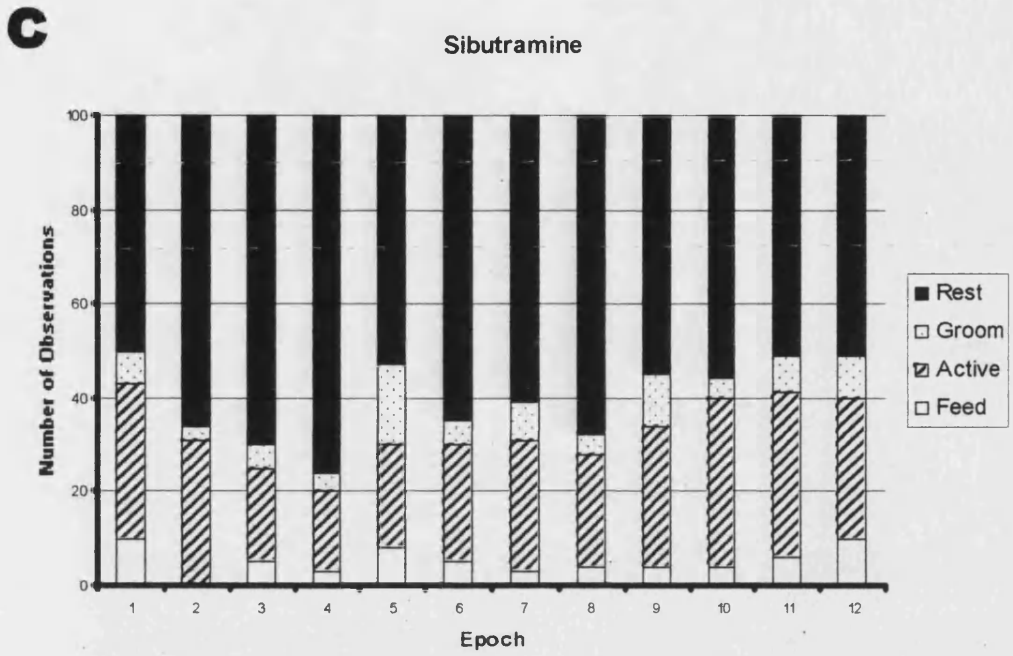
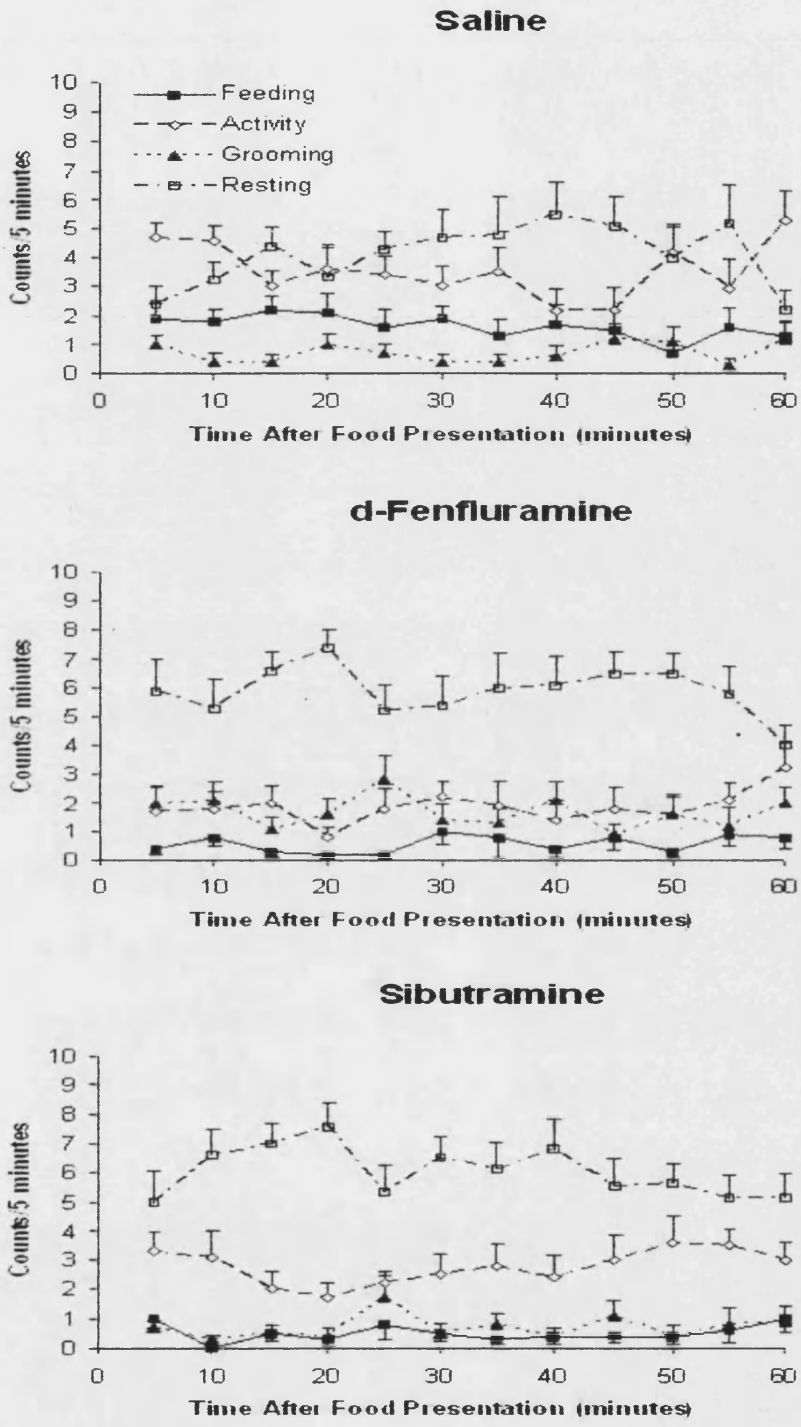


Figure 7.5.1(a-c)

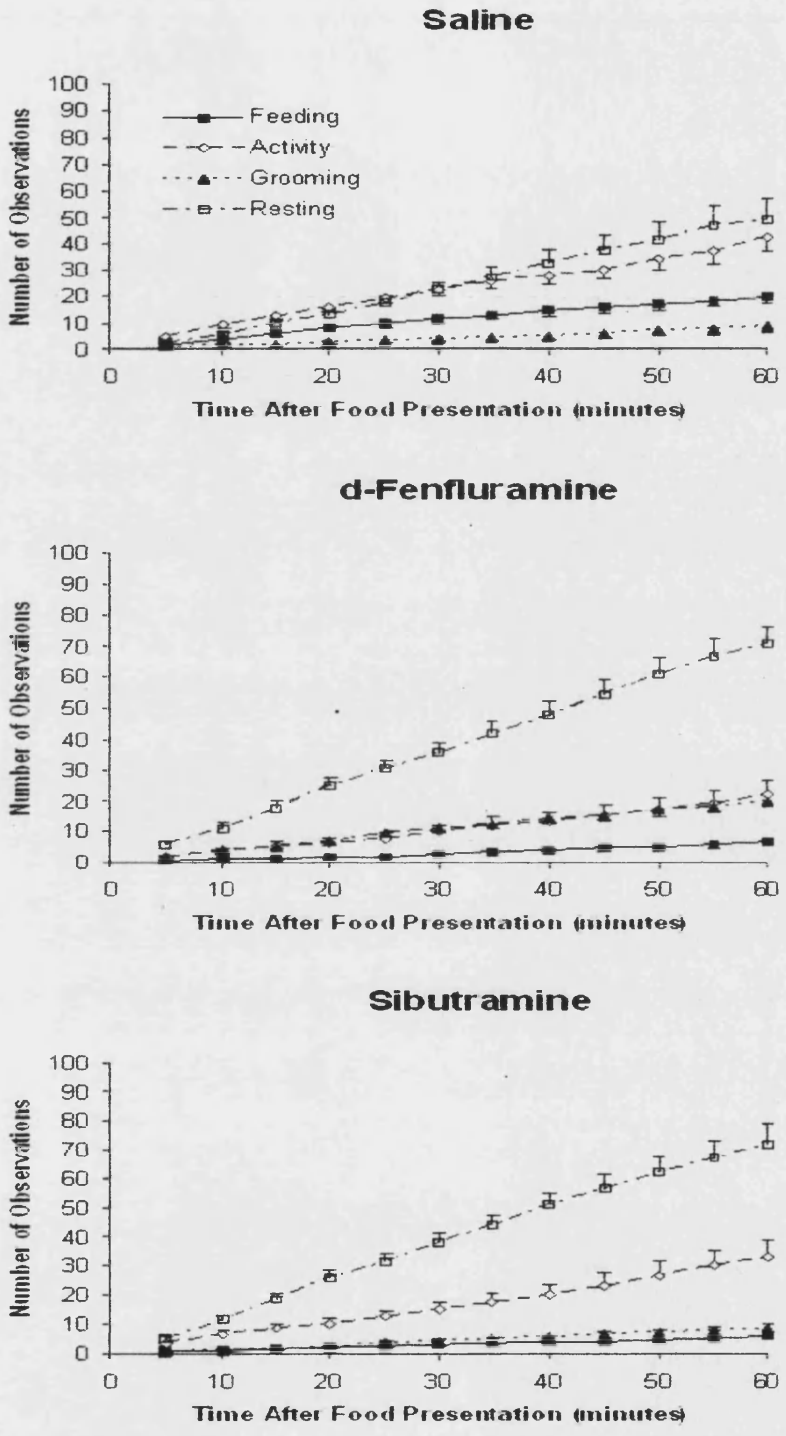
Behavioural profiles for animals observed 1 hour into the light phase after dosing with each of saline, 1.0 mg/kg *i.p.* *d*-fenfluramine and 1.67 mg/kg *i.p.* sibutramine. Observations are aggregates of scores from all animals in each epoch; epochs were of 5 minutes duration and $n = 10$ for every condition, with the same animals used in each case.

Figure 7.5.2 Observations of each behaviour during every epoch at CT1



Profiles indicating the level of observation for each of the four behavioural categories at CT1; each treatment is shown separately. Values are mean observations for each epoch ± standard error. $n = 16$

Figure 7.5.3 Cumulative observations of each behaviour at CT1



Profiles indicating the cumulative observation for each of the four behavioural categories at CT1; each treatment is shown separately. Values are mean cumulative totals after each epoch ± standard error, n = 10.

Figure 7.5.4a Behavioural profiles generated from BSS observations carried out at CT5 – the middle of the light phase.

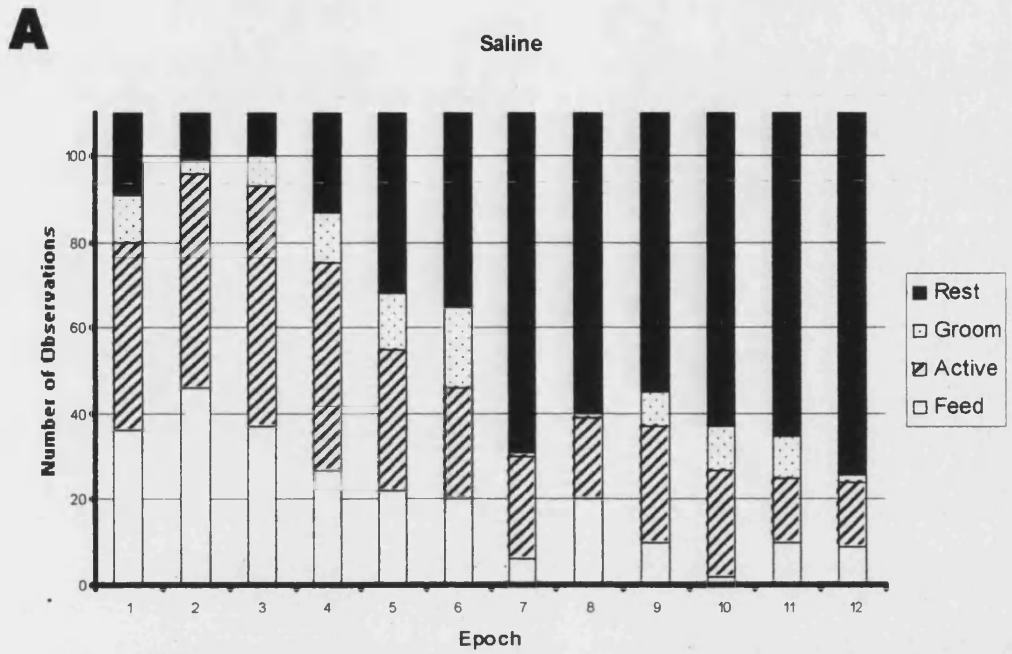


Figure 7.5.4b Behavioural profiles generated from BSS observations carried out at CT5 – the middle of the light phase.

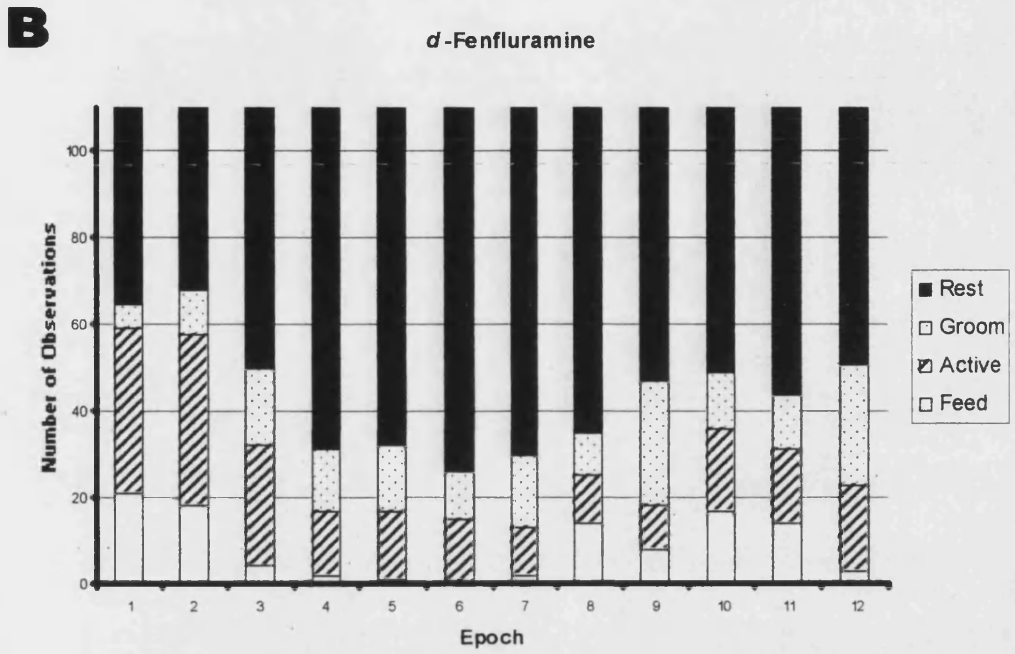


Figure 7.5.4c Behavioural profiles generated from BSS observations carried out at CT5 – the middle of the light phase.

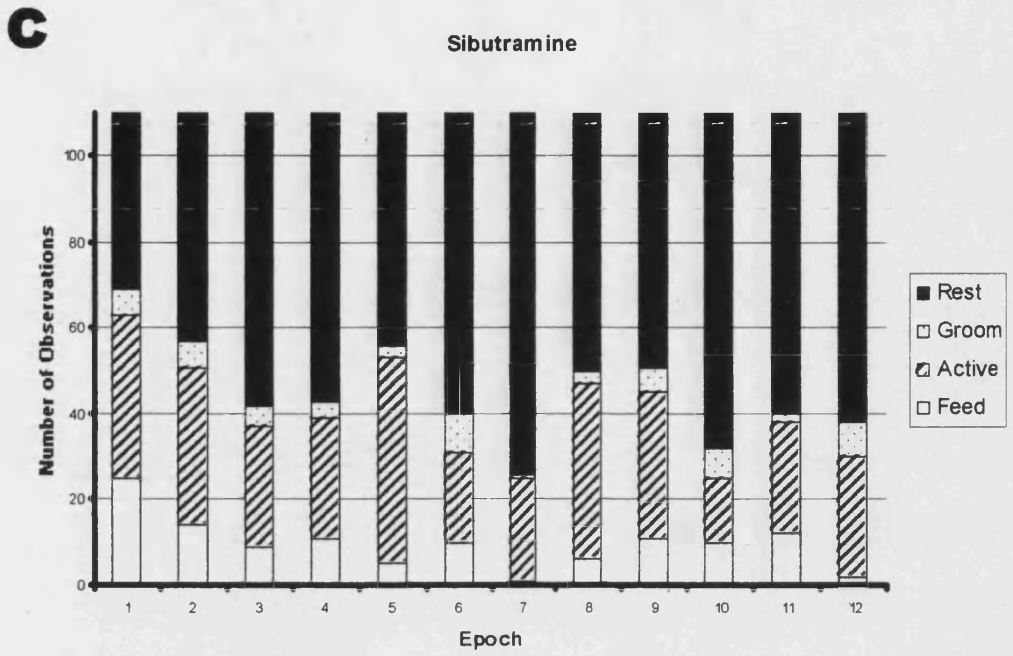
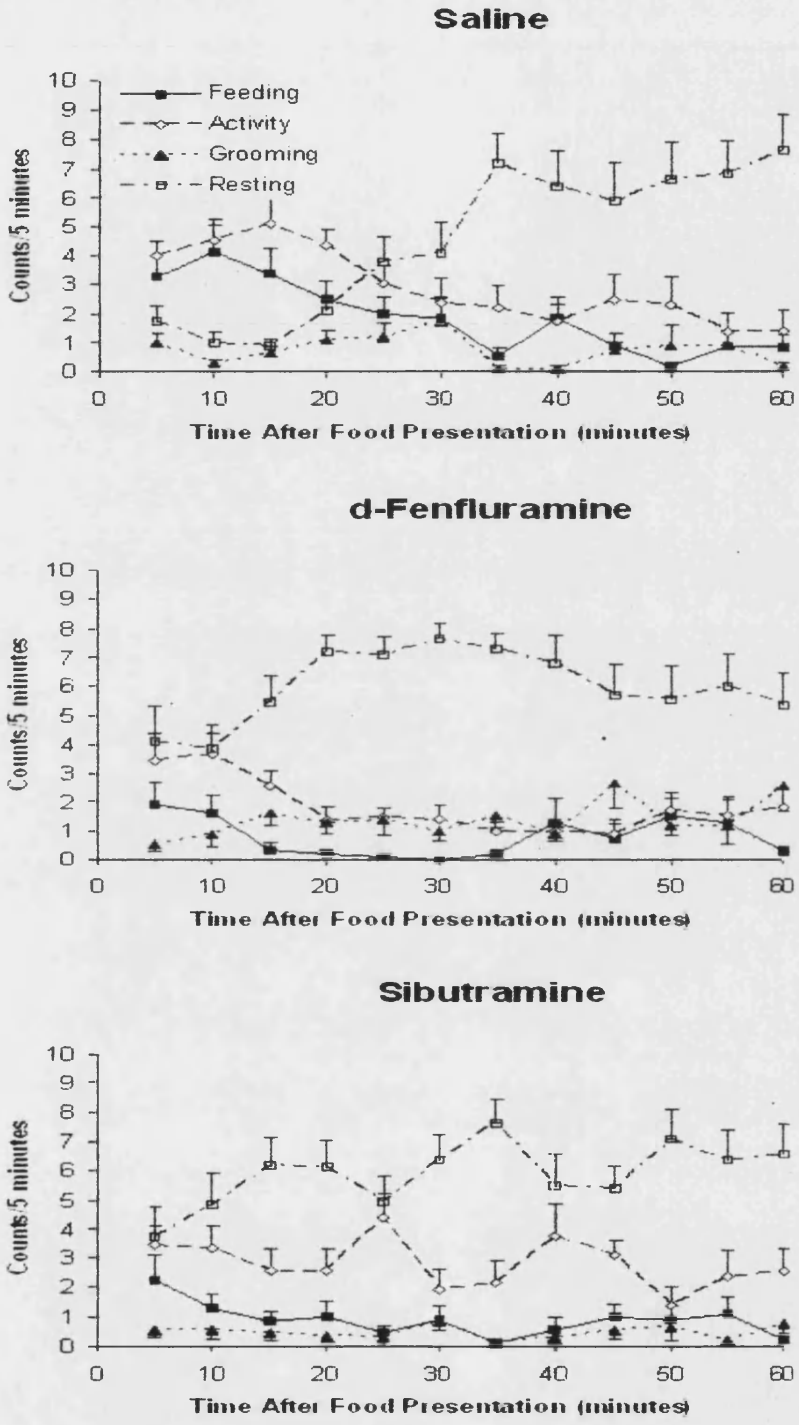


Figure 7.5.4(a-c)

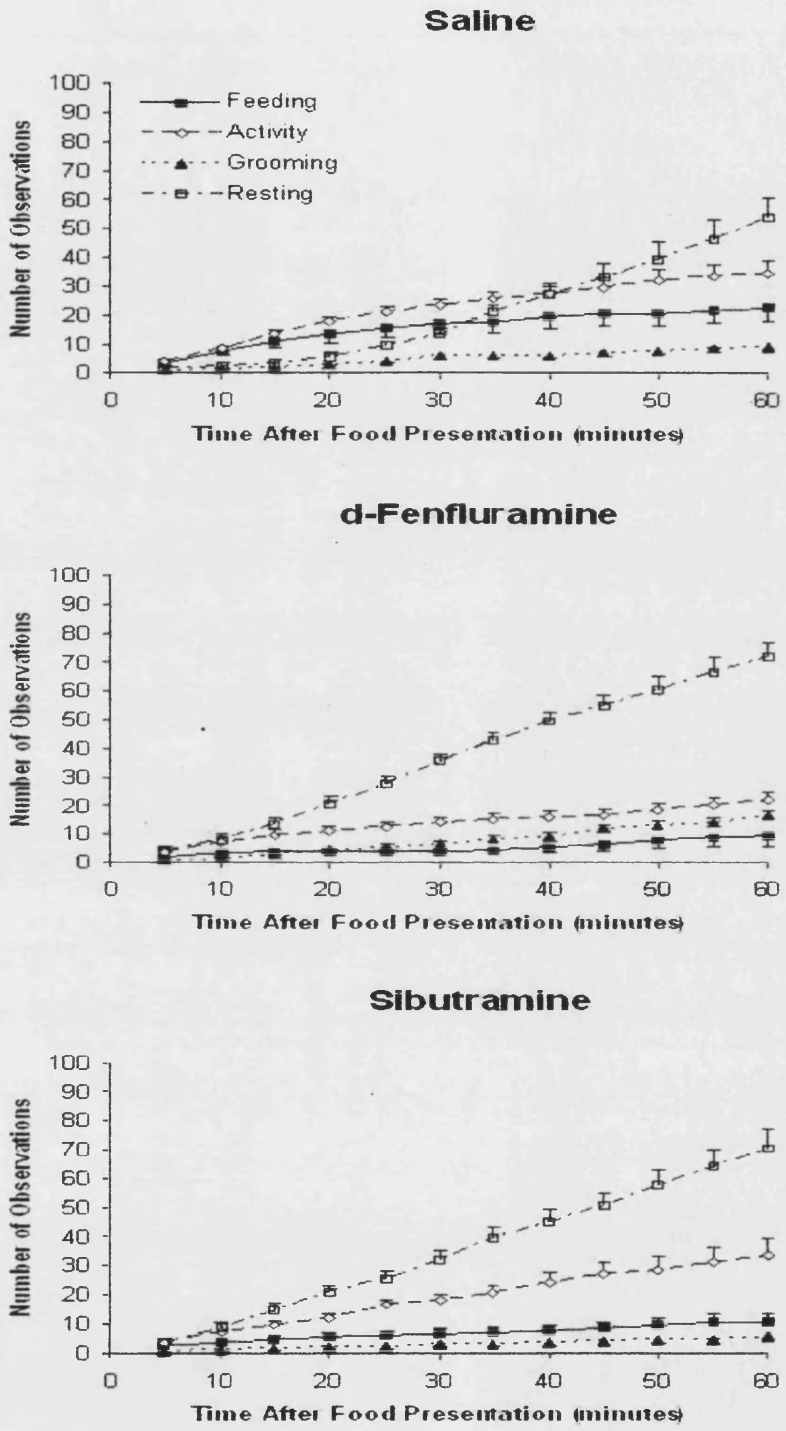
Behavioural profiles for animals observed 5 hours into the light phase after dosing with each of saline, 1.0 mg/kg *i.p. d*-fenfluramine and 1.67 mg/kg *i.p.* sibutramine. Observations are aggregates of scores from all animals in each epoch; epochs were of 5 minutes duration and $n = 11$ for every condition, with the same animals used in each case.

Figure 7.5.5 Observations of each behaviour during every epoch at CT5



Profiles indicating the level of observation for each of the four behavioural categories at CT5; each treatment is shown separately. Values are mean observations for each epoch ± standard error. *n* =

Figure 7.5.6 Cumulative observations of each behaviour at CT5



Profiles indicating the cumulative observation for each of the four behavioural categories at CT5; each treatment is shown separately. Values are mean cumulative totals after each epoch ± standard error, $N = 11$.

Figure 7.5.7a Behavioural profiles generated from BSS observations carried out at CT9 - late in the light phase.

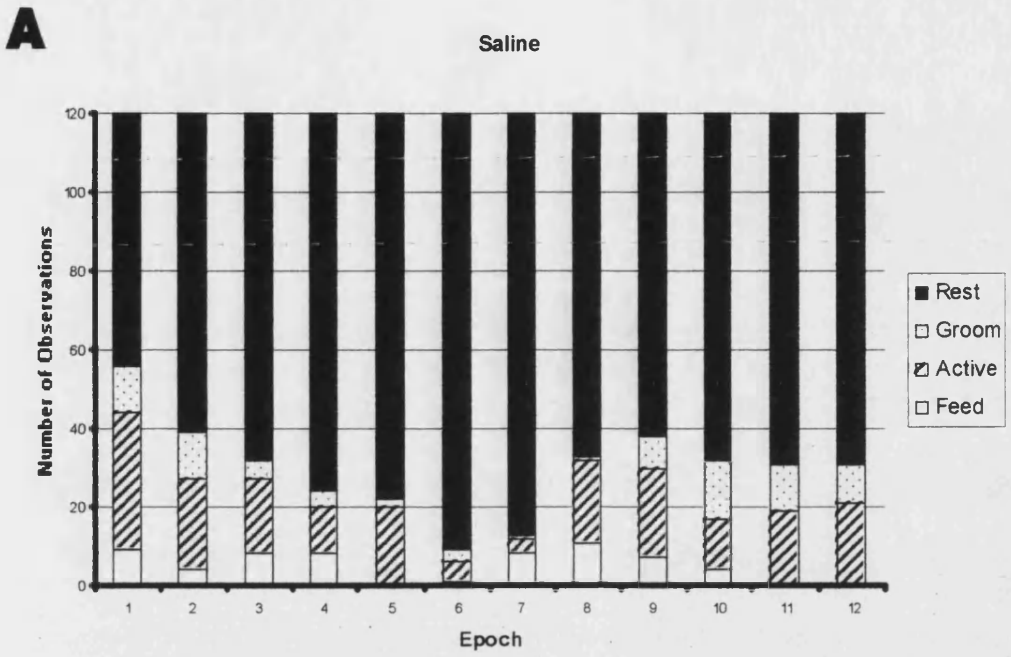


Figure 7.5.7b Behavioural profiles generated from BSS observations carried out at CT9 – late in the light phase.

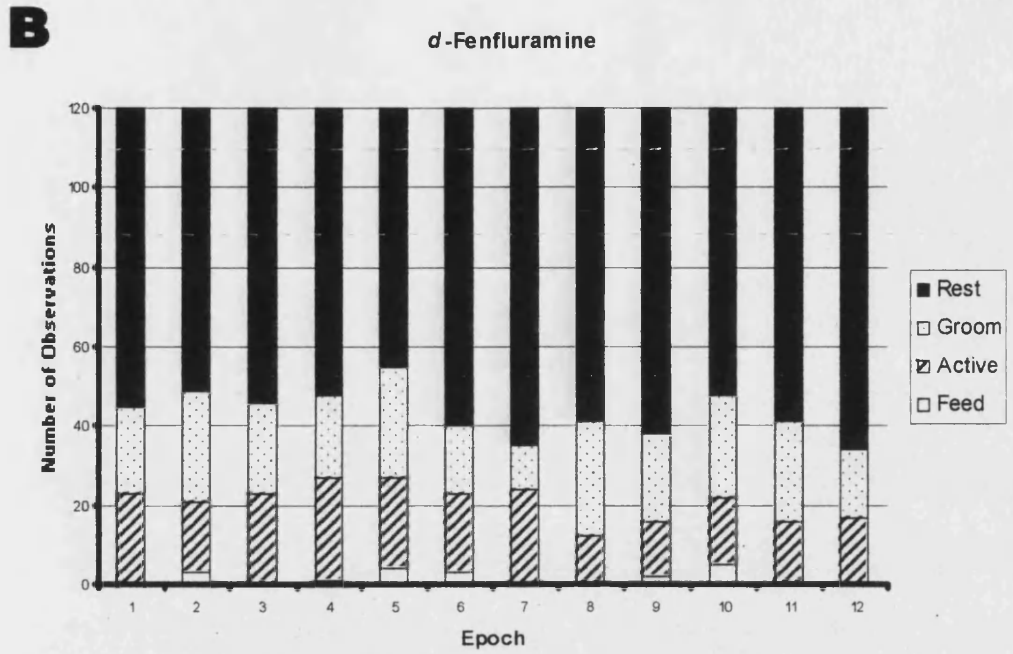


Figure 7.5.7c Behavioural profiles generated from BSS observations carried out at CT9 – late in the light phase.

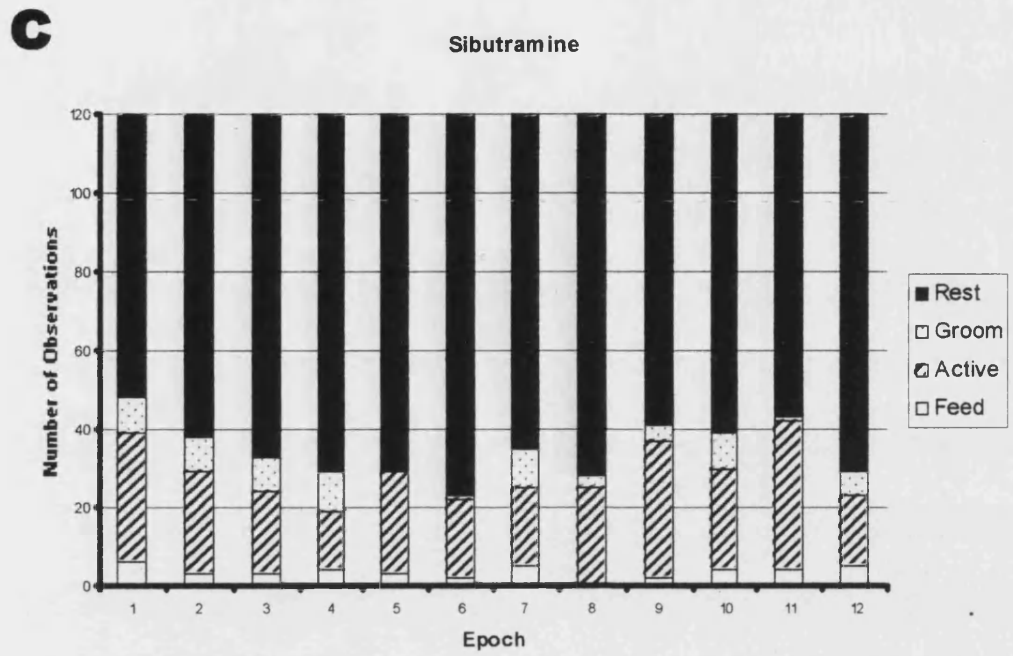
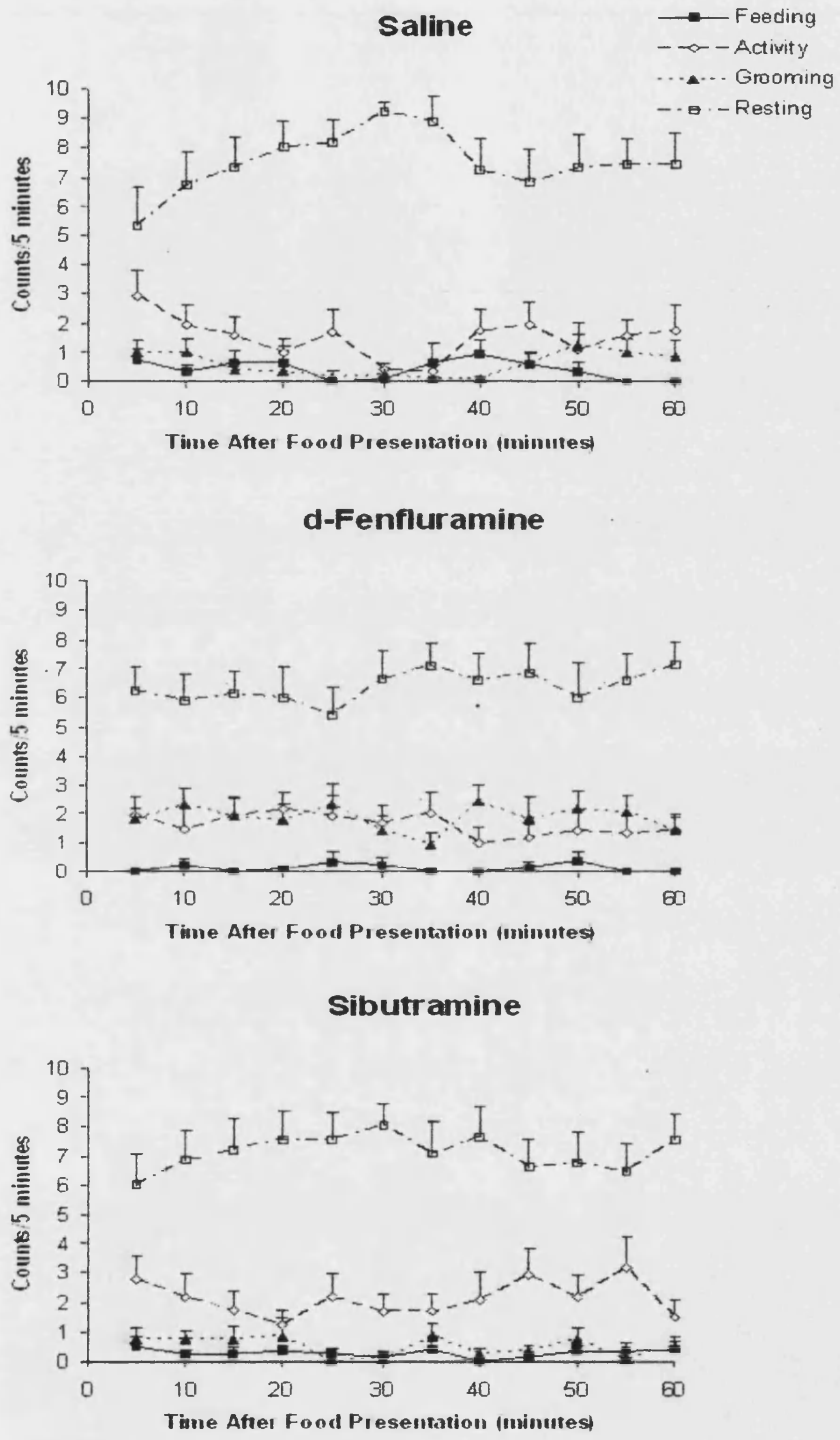


Figure 7.5.7(a-c)

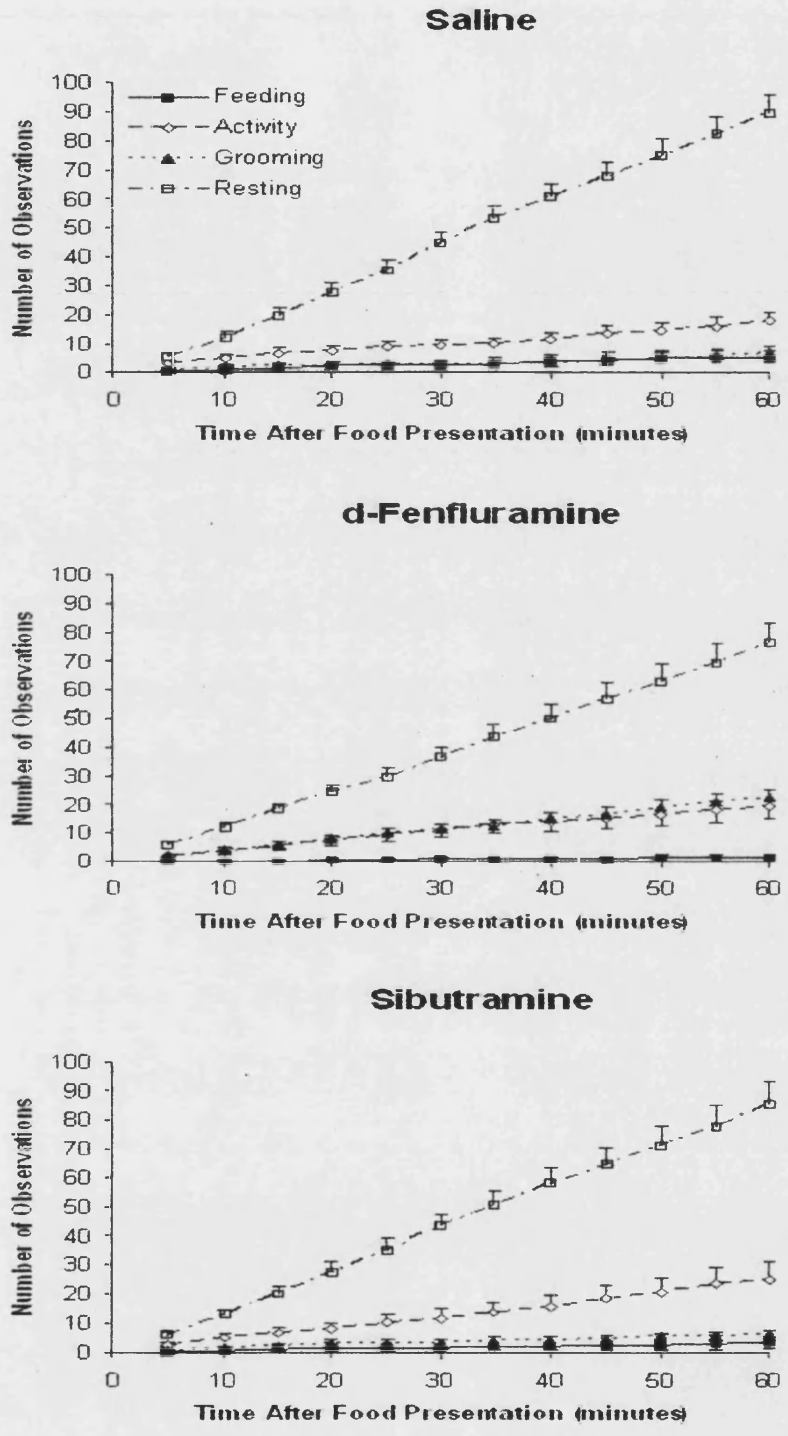
Behavioural profiles for animals observed 9 hours into the light phase after dosing with each of saline, 1.0 mg/kg *i.p.* *d*-fenfluramine and 1.67 mg/kg *i.p.* sibutramine. None of feeding, resting or activity were significantly affected by drug treatment at this time; grooming was significantly and robustly increased by treatment with *d*-fenfluramine however; this effect was observed 10 minutes into the test period. Observations are aggregates of scores from all animals in each epoch; epochs were of 5 minutes duration and $n = 12$ for every condition, with the same animals used in each case.

Figure 7.5.8 Observations of each behaviour during every epoch at CT9



Profiles indicating the level of observation for each of the four behavioural categories at CT9; each treatment is shown separately. Values are mean observations for each epoch ± standard error, n = 12.

Figure 7.5.9 Cumulative observations of each behaviour at CT9



Profiles indicating the cumulative observation for each of the four behavioural categories at CT9; each treatment is shown separately. Values are mean cumulative totals after each epoch ± standard error, $n = 12$.

Figure 7.5.10a Behavioural profiles generated from BSS observations carried out at CT13 – early in the dark phase.

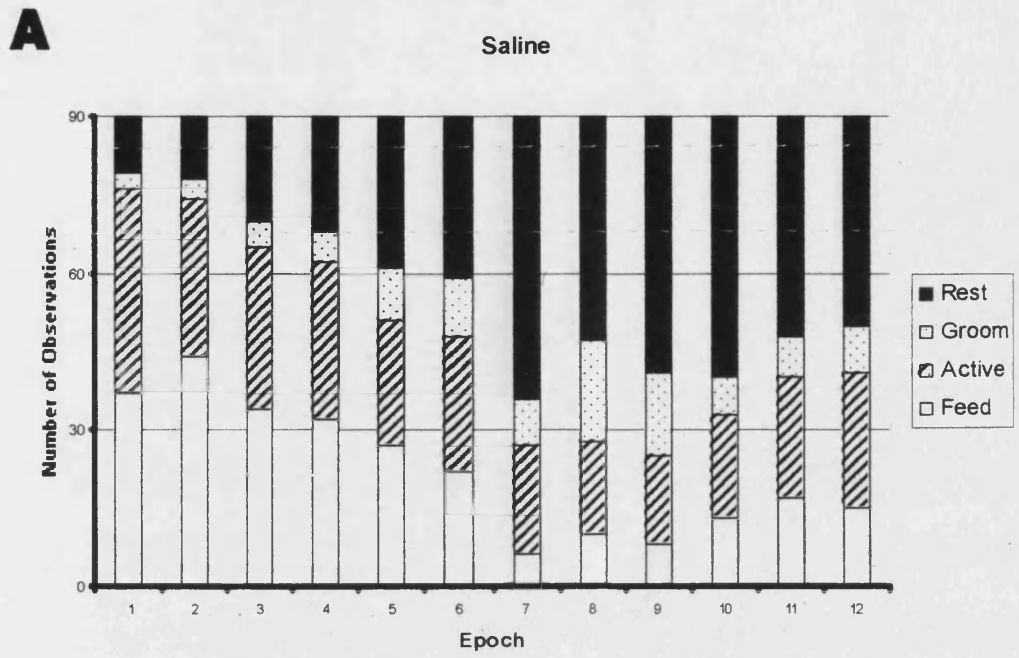


Figure 7.5.10b Behavioural profiles generated from BSS observations carried out at CT13 – early in the dark phase.

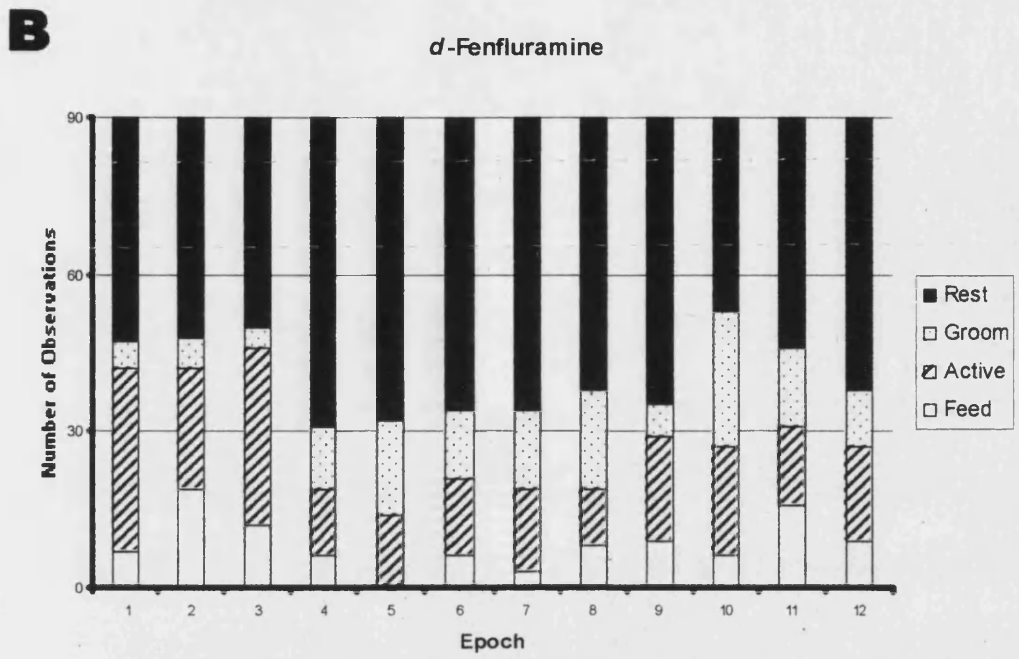


Figure 7.5.10c Behavioural profiles generated from BSS observations carried out at CT13 – early in the dark phase.

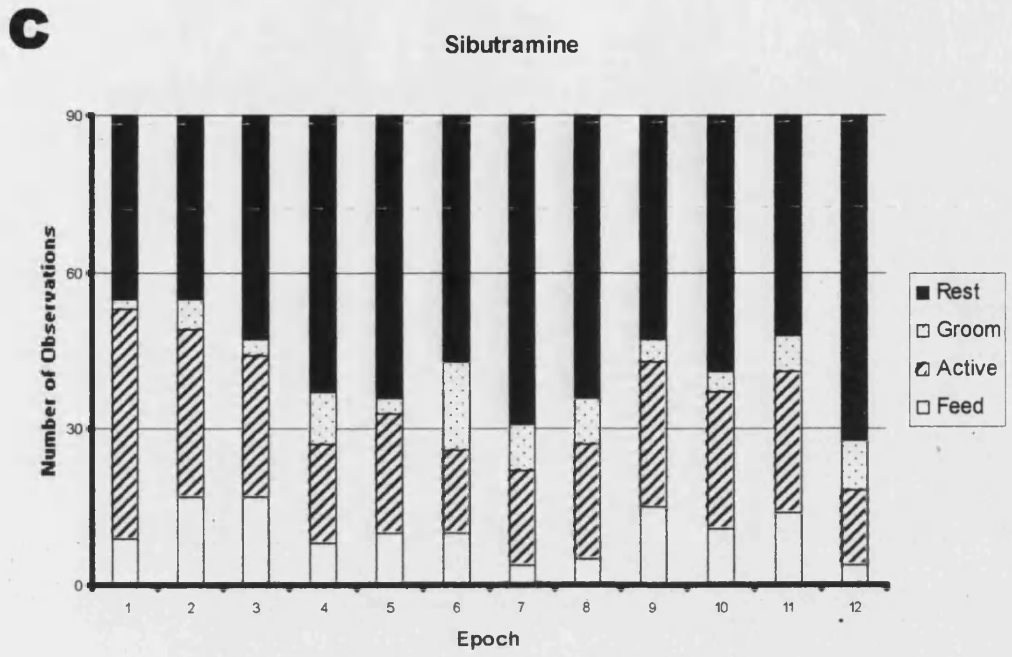
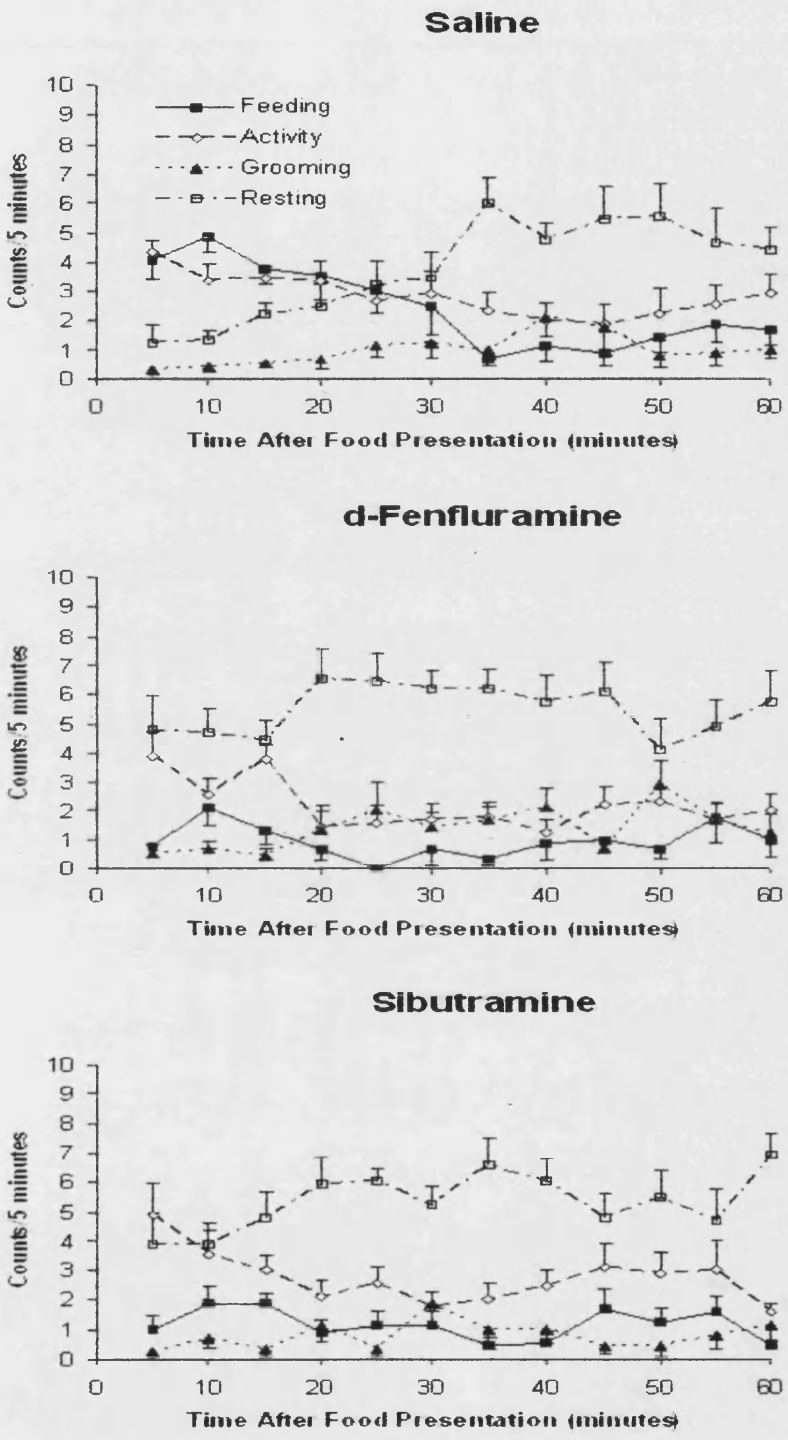


Figure 7.5.10(a-c)

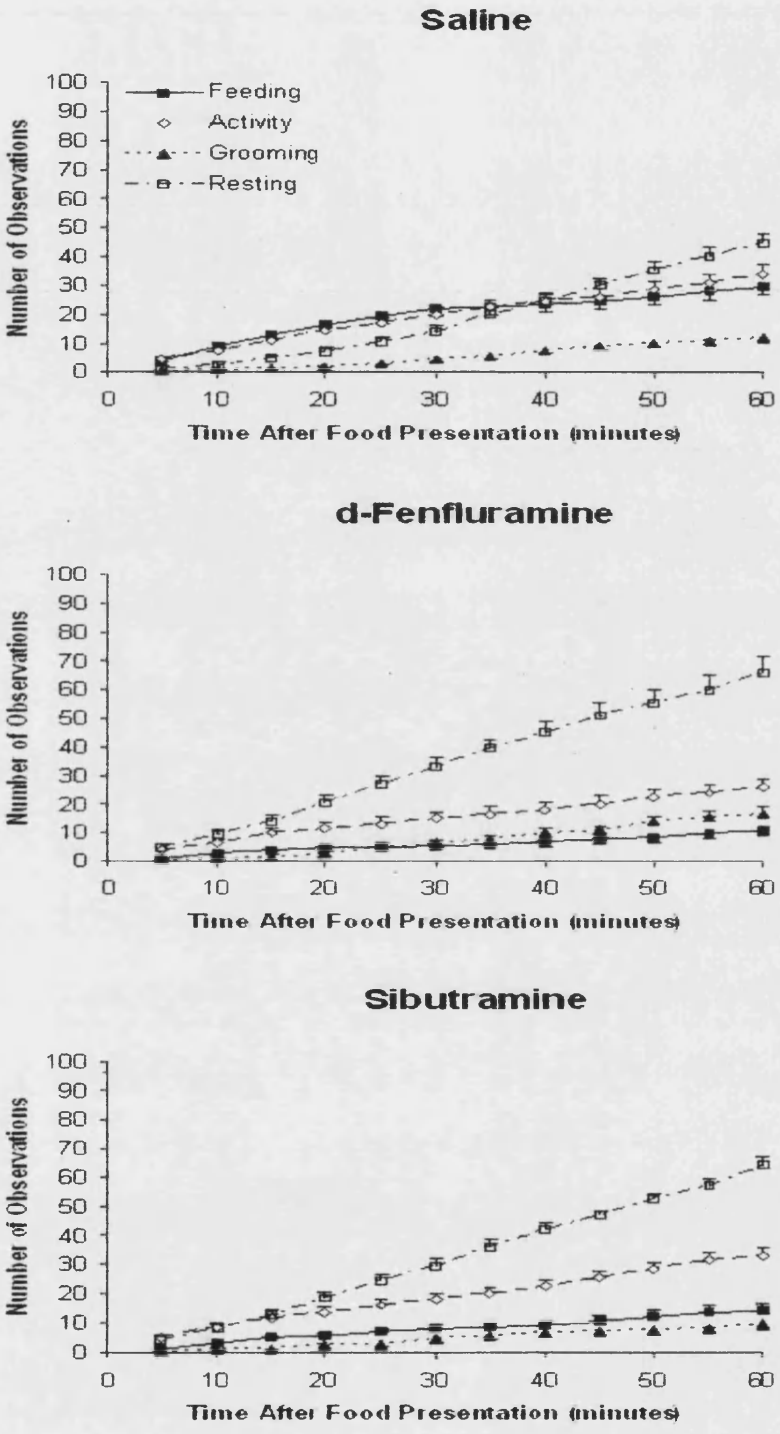
Behavioural profiles for animals observed 1 hour into the dark phase after dosing with each of saline, 1.0 mg/kg *i.p.* *d*-fenfluramine and 1.67 mg/kg *i.p.* sibutramine. Observations are aggregates of scores from all animals in each epoch; epochs were of 5 minutes duration and $n = 9$ for every condition, with the same animals used in each case.

Figure 7.5.11 Observations of each behaviour during every epoch at CT13



Profiles indicating the level of observation for each of the four behavioural categories at CT13; each treatment is shown separately. Values are mean observations for each epoch ± standard error.

Figure 7.5.12 Cumulative observations of each behaviour at CT13



Profiles indicating the cumulative observation for each of the four behavioural categories at CT13; each treatment is shown separately. Values are mean cumulative totals after each epoch ± standard error, $n = 6$.

Figure 7.5.13a Behavioural profiles generated from BSS observations carried out at CT17 – in the middle of the dark phase.

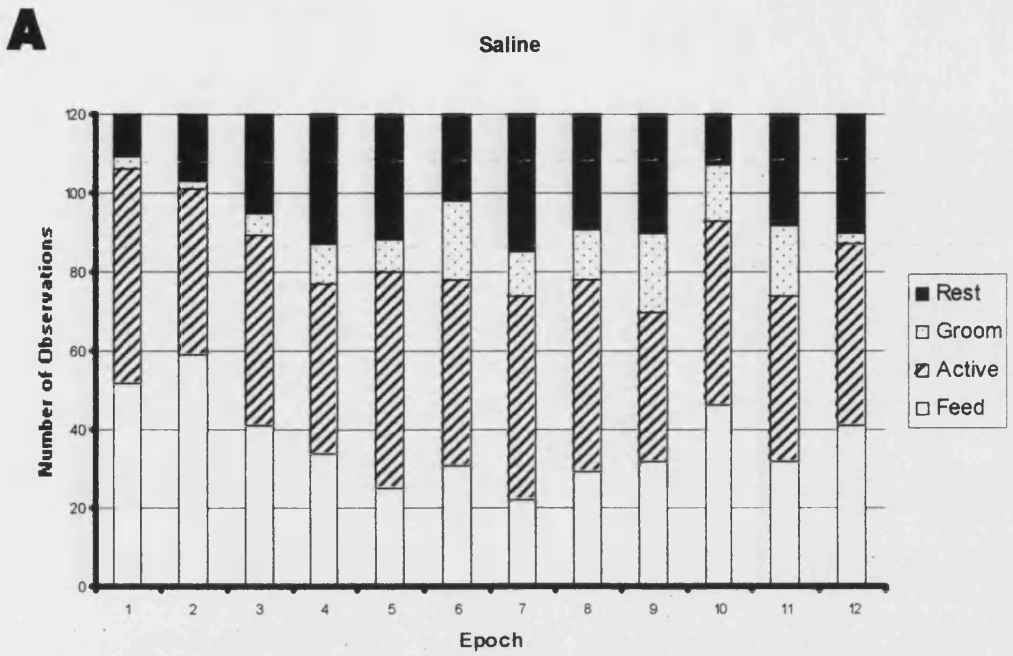


Figure 7.5.13b Behavioural profiles generated from BSS observations carried out at CT17 – in the middle of the dark phase.

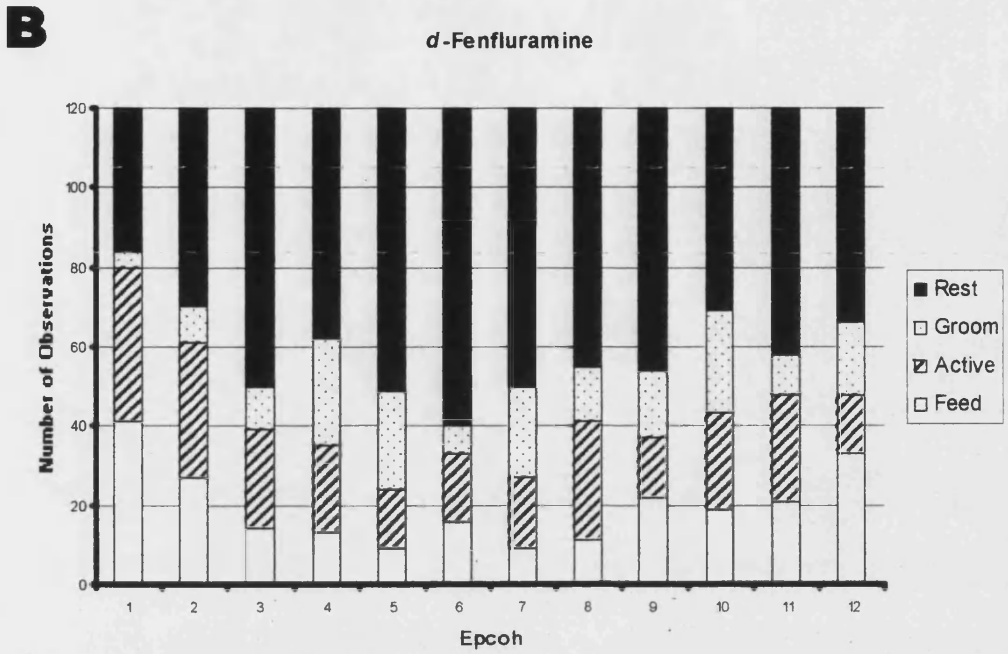


Figure 7.5.13c Behavioural profiles generated from BSS observations carried out at CT17 – in the middle of the dark phase.

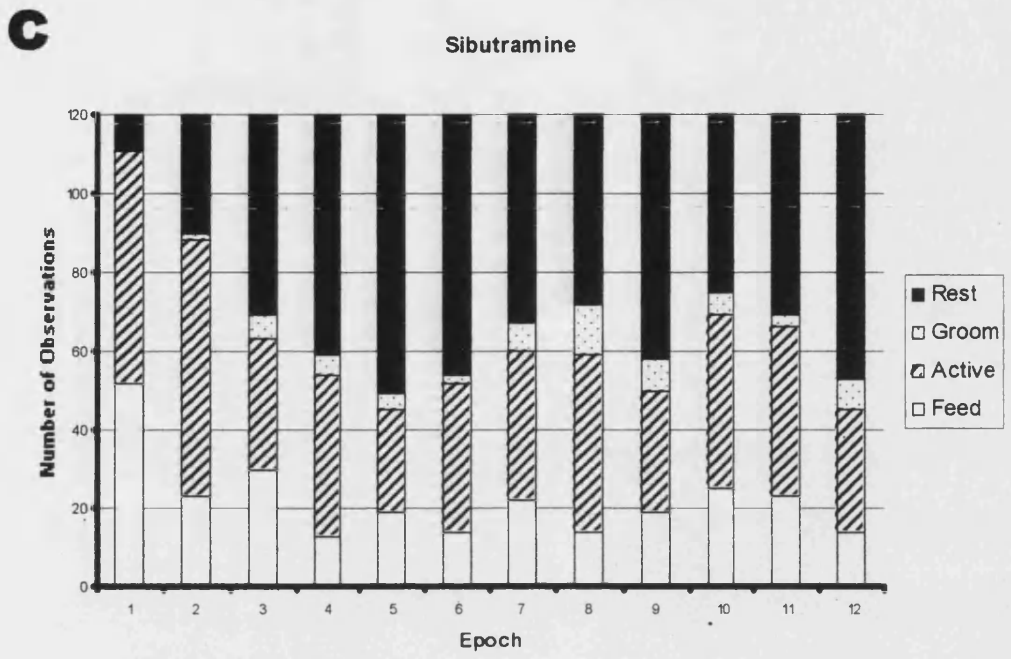
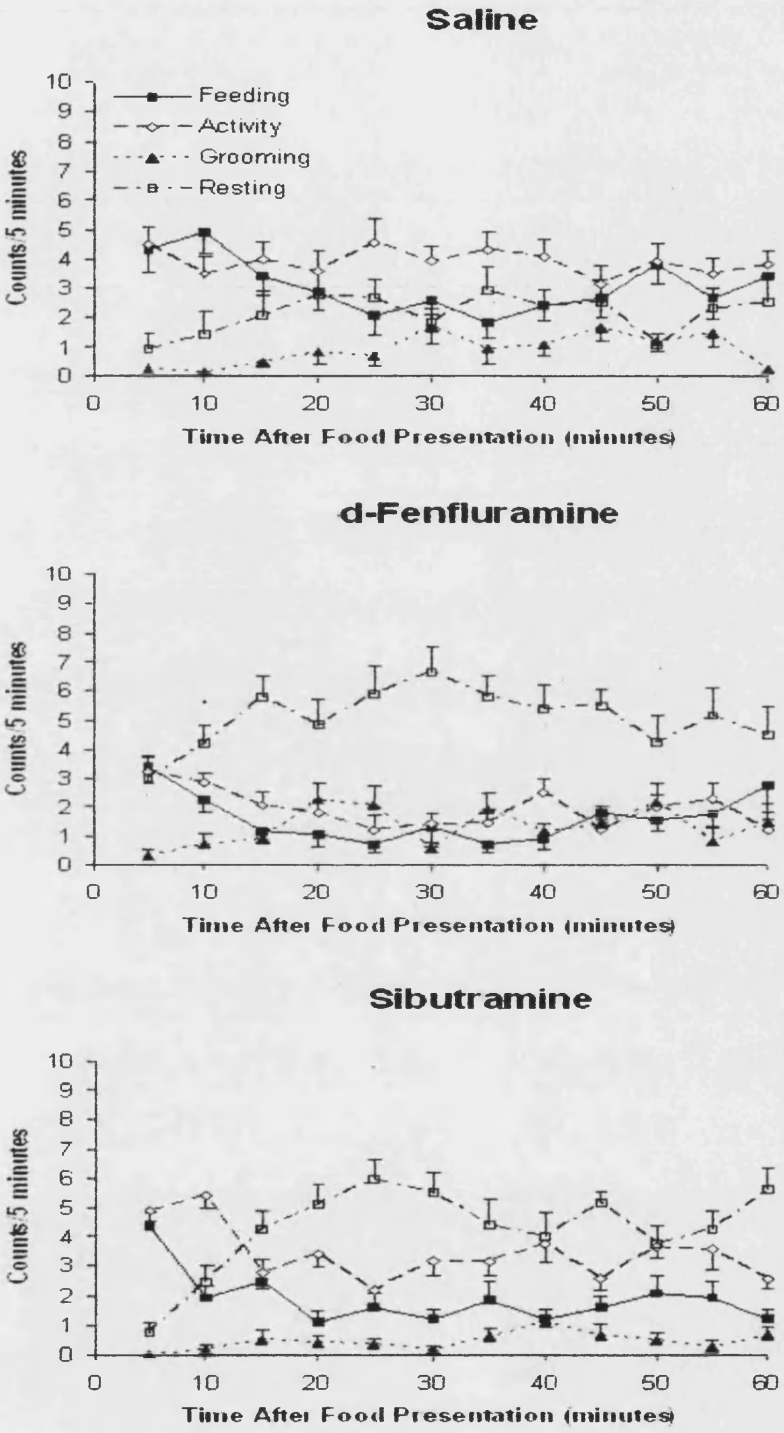


Figure 7.5.13(a-c)

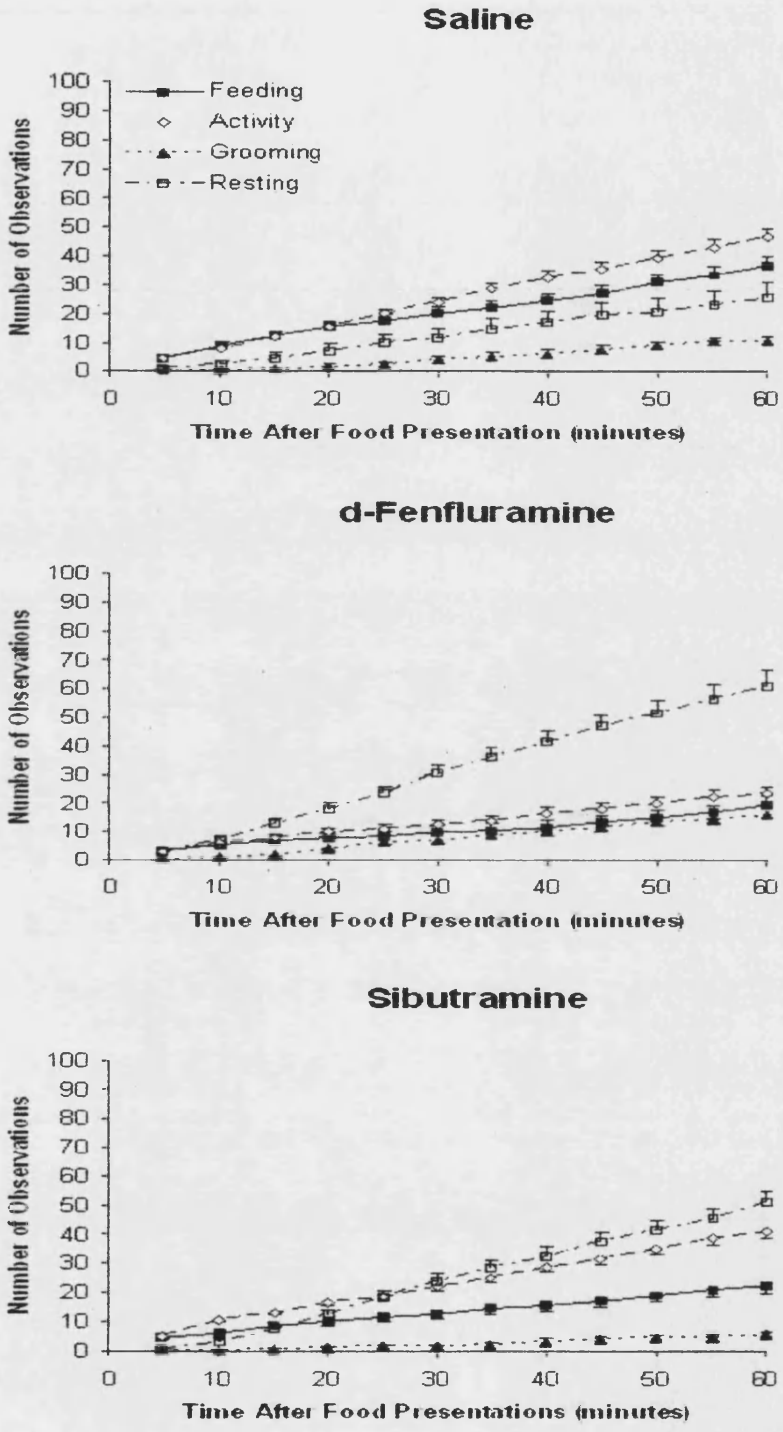
Behavioural profiles for animals observed 5 hours into the dark phase after dosing with each of saline, 1.0 mg/kg *i.p. d*-fenfluramine and 1.67 mg/kg *i.p.* sibutramine. Observations are aggregates of scores from all animals in each epoch; epochs were of 5 minutes duration and $n = 12$ for every condition, with the same animals used in each case.

Figure 7.5.14 Observations of each behaviour during every epoch at CT17



Profiles indicating the level of observation for each of the four behavioural categories at CT17; each treatment is shown separately. Values are mean observations for each epoch ± standard error, *n* = 12.

Figure 7.5.15 Cumulative observations of each behaviour at CT17



Profiles indicating the cumulative observation for each of the four behavioural categories at CT17; each treatment is shown separately. Values are mean cumulative totals after each epoch ± standard error, $n = 12$.

Figure 7.5.16a Behavioural profiles generated from BSS observations carried out at CT21 – late in the dark phase.

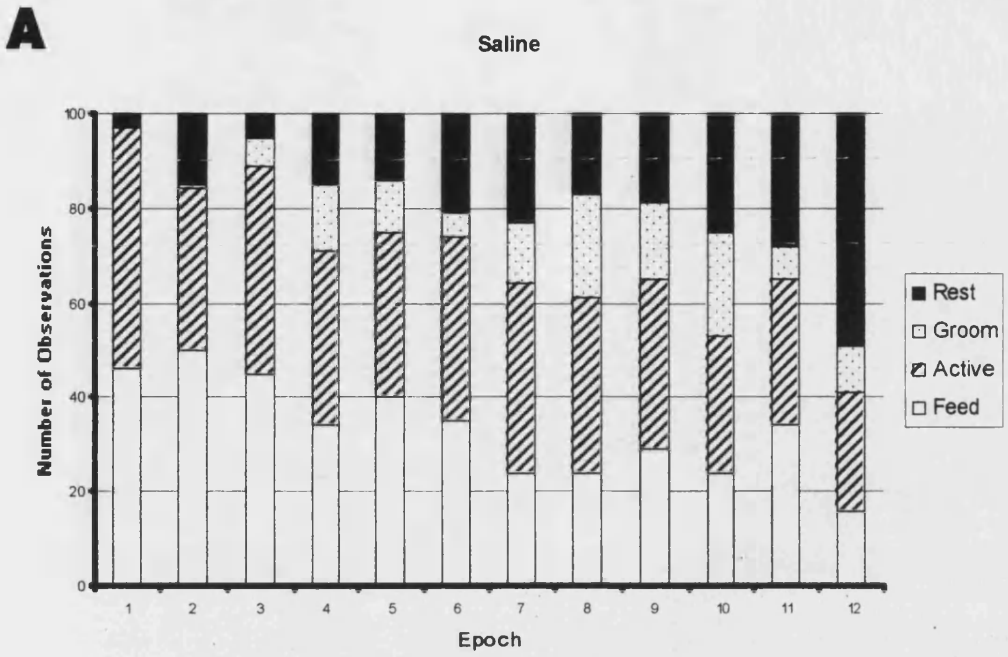


Figure 7.5.16b Behavioural profiles generated from BSS observations carried out at CT21 – late in the dark phase.

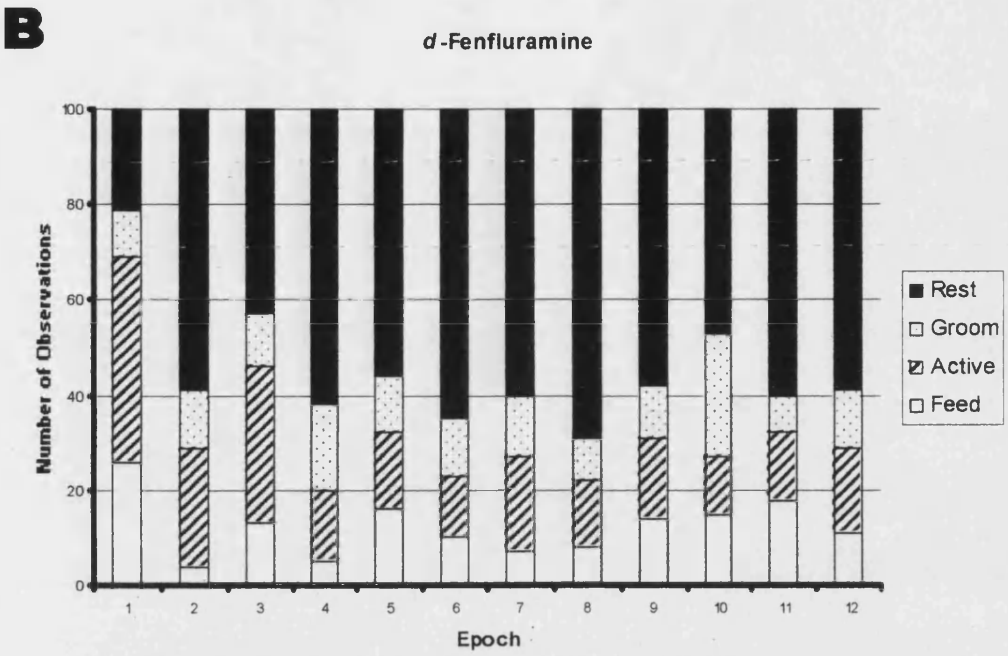


Figure 7.5.16c Behavioural profiles generated from BSS observations carried out at CT21 – late in the dark phase.

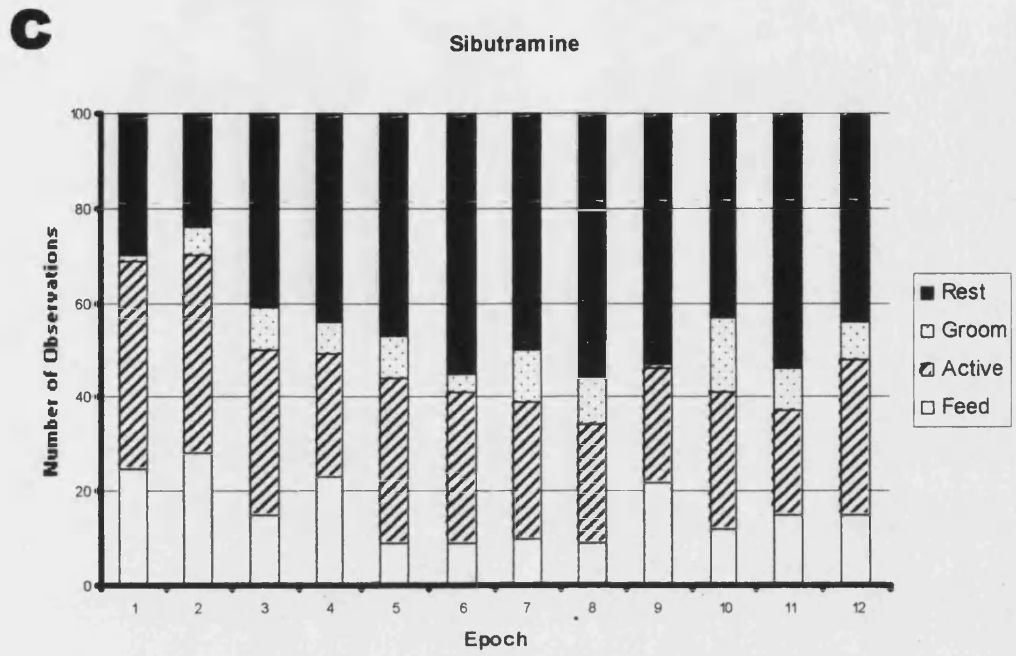
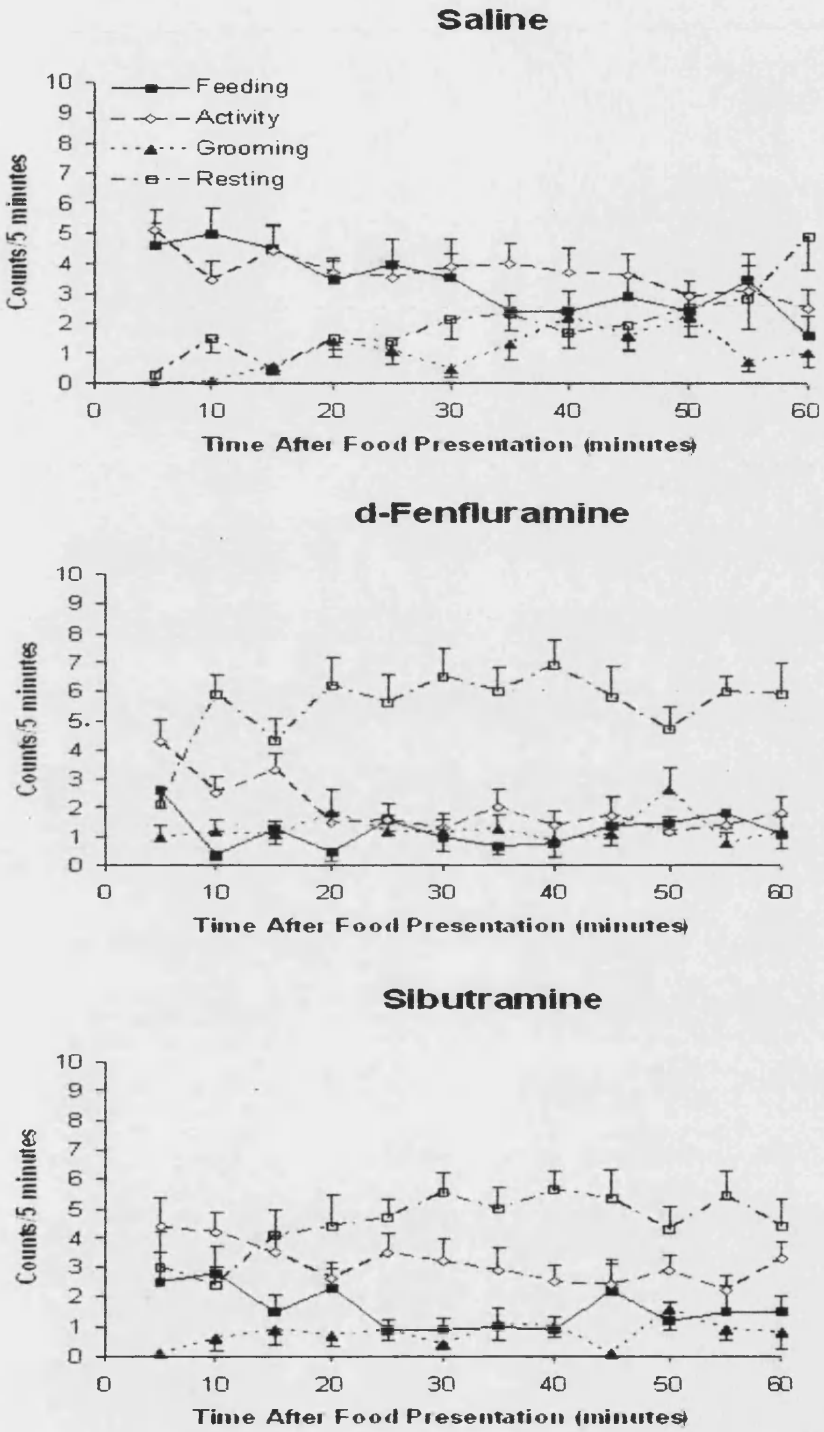


Figure 7.5.16(a-c)

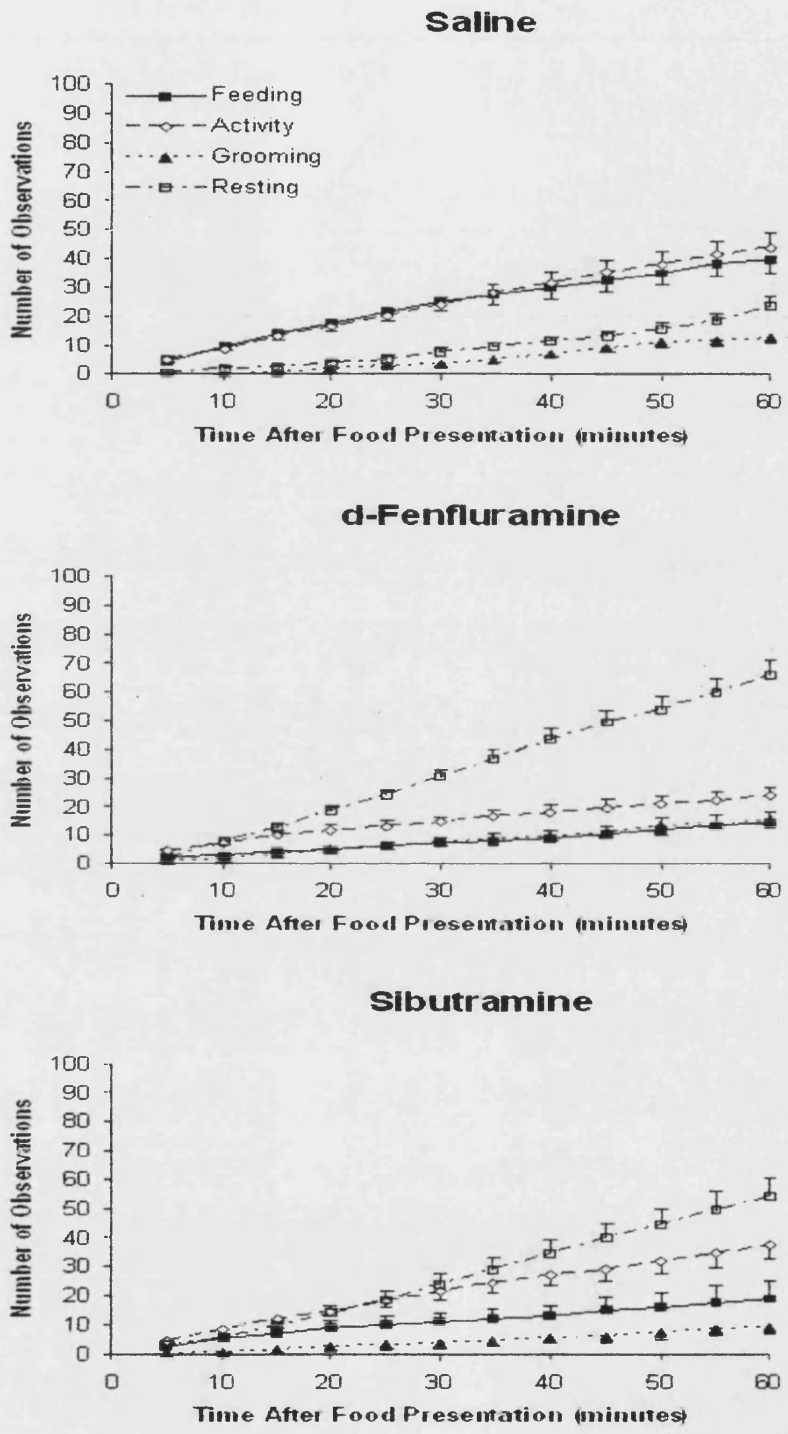
Behavioural profiles for animals observed 9 hours into the dark phase after dosing with each of saline, 1.0 mg/kg *i.p.* *d*-fenfluramine and 1.67 mg/kg *i.p.* sibutramine. Observations are aggregates of scores from all animals in each epoch; epochs were of 5 minutes duration and $n = 10$ for every condition, with the same animals used in each case.

Figure 7.5.17 Observations of each behaviour during every epoch at CT21



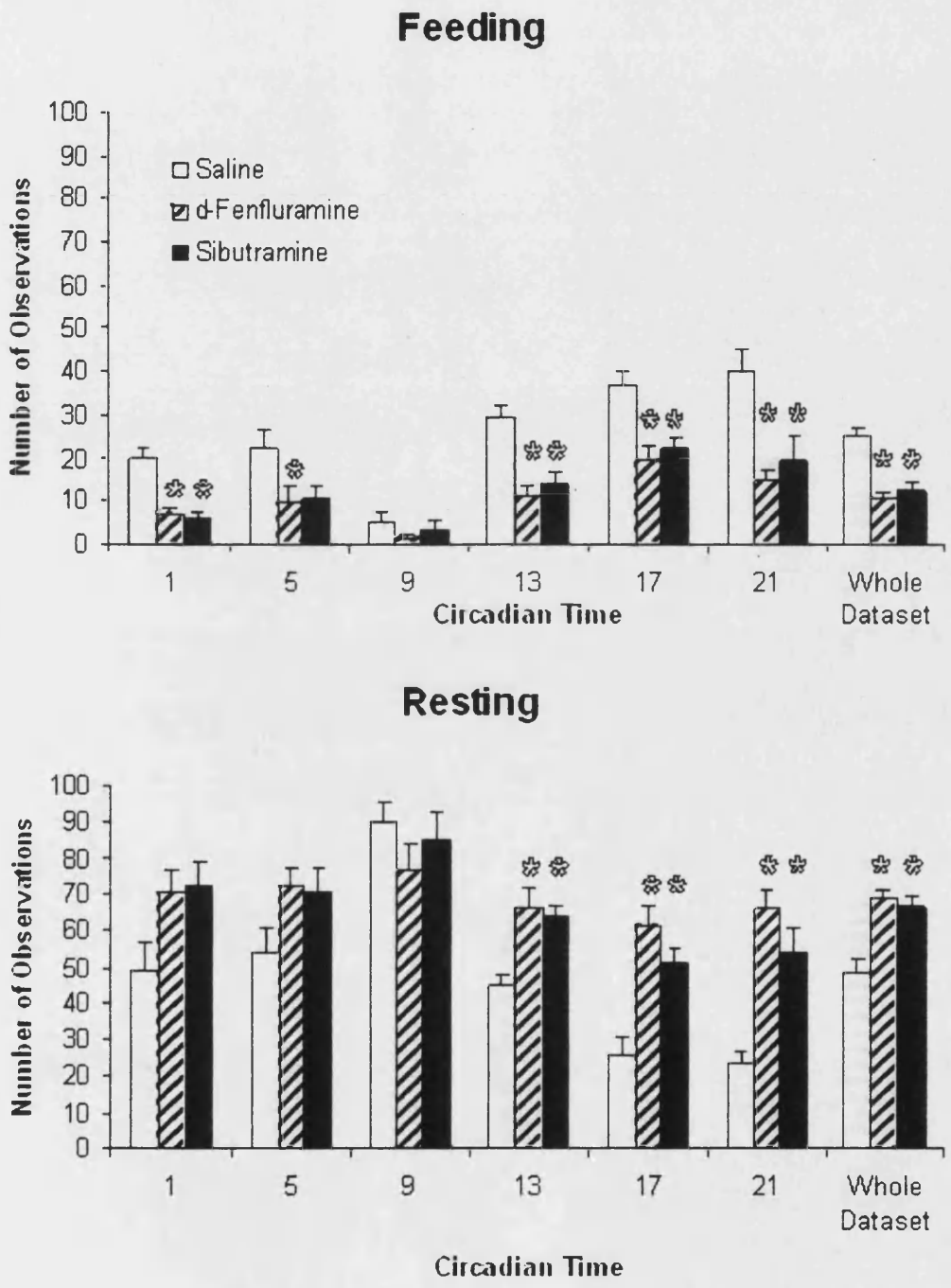
Profiles indicating the level of observation for each of the four behavioural categories at CT21; each treatment is shown separately. Values are mean observations for each epoch ± standard error, $n = 10$.

Figure 7.5.18 Cumulative observations of each behaviour at CT21



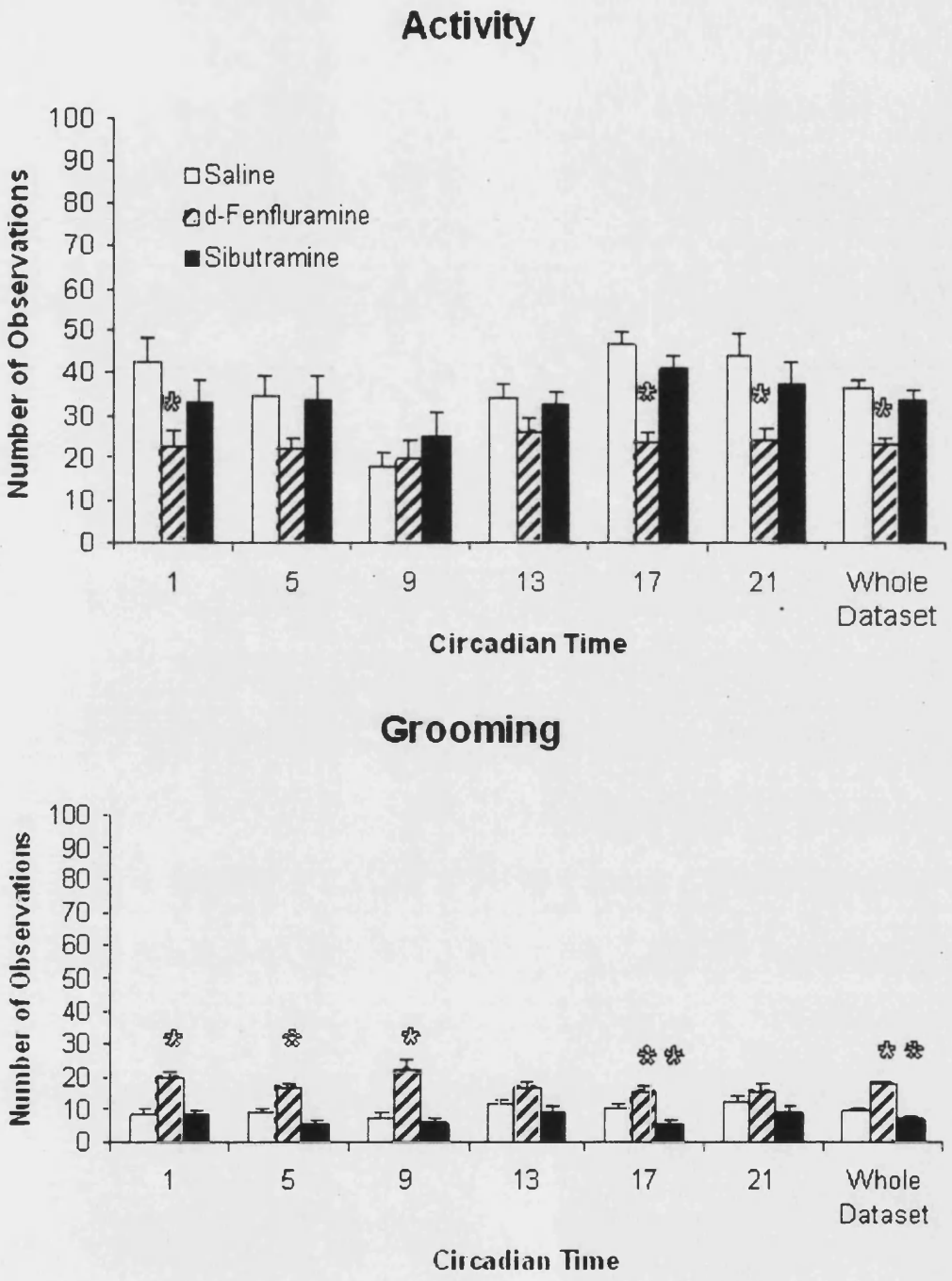
Profiles indicating the cumulative observation for each of the four behavioural categories at CT21; each treatment is shown separately. Values are mean cumulative totals after each epoch ± standard error. $n = 10$.

Figure 7.5.19a Representation of the effects of *d*-fenfluramine and sibutramine on feeding and resting behaviour over the circadian cycle.



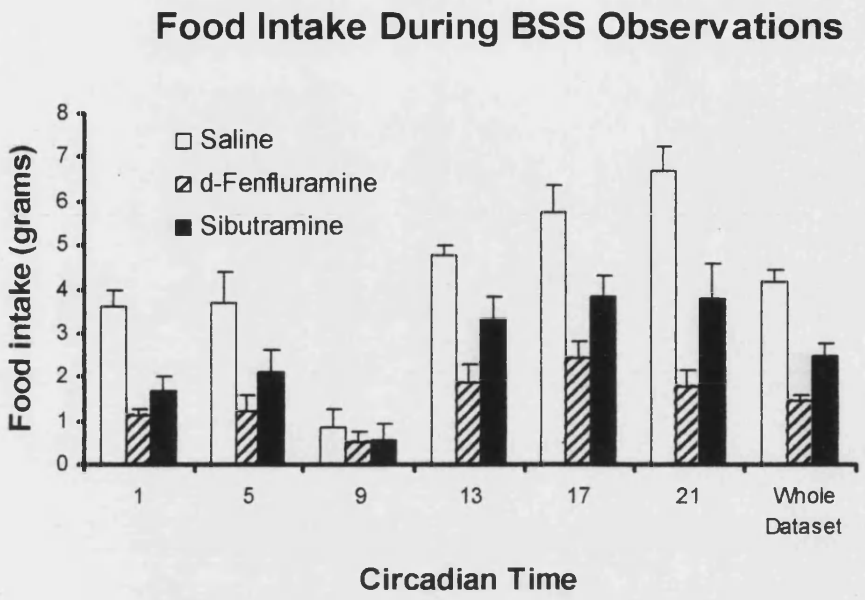
❀ $p < 0.05$ compared with saline treatment at a similar circadian time

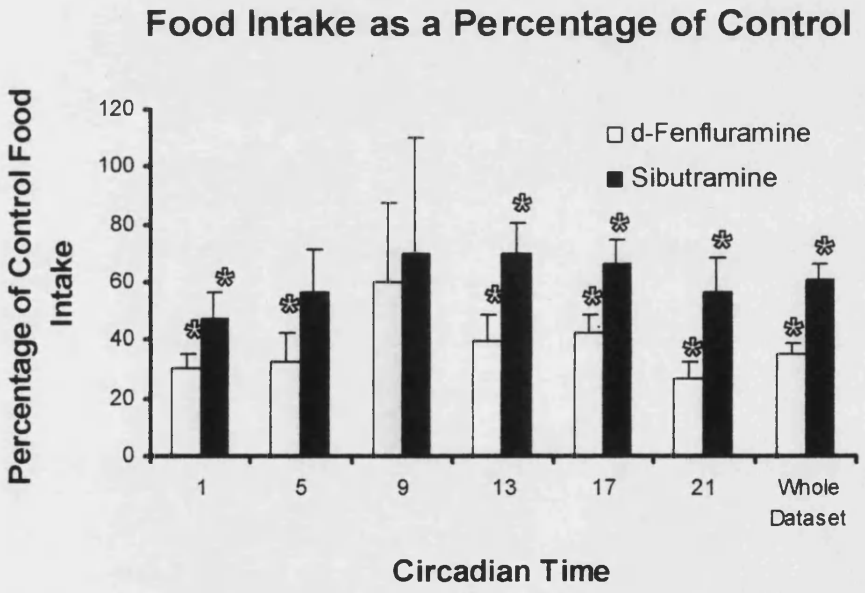
Figure 7.5.19b Representation of the effects of *d*-fenfluramine and sibutramine on activity and grooming behaviour over the circadian cycle.



❀ $p < 0.05$ compared with saline treatment at a similar circadian time.

Figure 7.5.20 Food intake across the circadian cycle, and the degree of reduction caused by sibutramine or *d*-fenfluramine at each point.





❀ $p < 0.05$ compared with saline treatment at a similar circadian time.

Table 7.5.1 A summary of how soon into observations the drug-induced changes on behaviour were noted with respect to saline, displayed by circadian time.

Behaviour	Drug	Circadian Time						
		1	5	9	13	17	21	Across All
Feeding	d-fen	***5-60***	***15-60***	N/A	***5-60***	***10-60***	***10-60***	***5-60***
	Sibutramine	***10-60***	***15-50***	N/A	***5-60***	***10-60***	***15-60***	***5-60***
Activity	d-fen	***5-60***	***20-50***	N/A	N/A	***15-60***	***30-60***	***5-60***
	Sibutramine	***20-25***	20	N/A	N/A	10	N/A	N/A
Grooming	d-fen	10-60	45-60	10-60	N/A	20-25; 50; 60	5-20	5-60
	Sibutramine	N/A	N/A	N/A	N/A	***50-60***	N/A	***50-60***
Resting	d-fen	5-45	15-50	N/A	5-60	5-60	10-60	5-60
	Sibutramine	10-50	10-50	N/A	10-60	25-60	15-60	5-60

Footnotes:

- Italicised and bolded entries represent a decrease in observations compared to the saline condition. Other entries represent an increase relative to controls
- N/A represents no significant difference at any point.
- Single numbers represent significance at one point only.
- Numbers separated by dashes indicate a period of significant difference.
- Semi-colons are used to separate non-consecutive instances.

8.0 General Discussion

What can we learn from taking the results described above taken not individually or in isolation but together – and in the context of the published literature? What remains to be done in order to further clarify the nature of the impact of circadian variation on treatment with these two drugs, sibutramine and *d*-fenfluramine?

To tackle the second point first, it would have been interesting to have been able to monitor circadian changes not only in the behavioural response to sibutramine and *d*-fenfluramine but also in neurochemical and pharmacokinetic responses to these drugs, as well as binding data for relevant regions of the brain. Originally it had been the plan to carry out concurrent examinations of changes in neurochemistry caused by both compounds over the circadian cycle, with a view to probing the hypothalamus using microdialysis. However it proved impossible to undertake all sets of experiments in parallel; since the behavioural experiments were further advanced the neurochemistry had to be set aside. It remains an interesting question. Is circadian variation in the efficacy of the drugs simply correlated with basal, or tissue, neurotransmitter levels? Maybe it is related to the degree of change in transmitter levels induced by the drugs? Or perhaps with the maximal level reached? How do these parameters vary across the circadian cycle? It would be interesting to investigate paradigms of food deprivation and dosing, comparable to those used with the BSS studies and circadian locomotor activity work, whilst measuring extracellular neurotransmitter levels in feeding-associated nuclei of the hypothalamus by microdialysis. Several dialysis studies have been done with a view to increasing understanding of feeding behaviour and satiation (*e.g.* Fetissov *et al.*, 2000; Helm *et al.*, 2003; reviews by Westerink, 1995, and Rueter *et al.*, 1997) and these generally match an increase in extracellular hypothalamic 5-HT to feeding behaviour (Rueter *et al.*, 1997). Similarly dialysis studies have informed understanding of the roles played by NA and dopamine in feeding and the 24 hour rhythmicity of NA and 5-HT in feeding and other behaviours (Rueter *et al.*, 1997; Westerink, 1995). Detailed studies looking at the rhythmic variation in the efficacy and neurochemical impact of sibutramine or *d*-fenfluramine have not yet been carried out across the circadian cycle.

Perhaps treatment with either drug has a greater or lesser effect on transmitter levels – in real or percentage terms – at different times of day? One can imagine the magnitude of effect observed with a releasing agent like *d*-fenfluramine varying with the levels of 5-HT stored within the neurons, a phenomenon which is known to exhibit circadian rhythmicity (*e.g.* Martin and Redfern, 1997); it could be hypothesised that the more transmitter is stored the more can be released by the drug. On the other hand, the sheer scale of the response to fenfluramine, with around a 2500% increase in basal levels (*e.g.* Gundlah *et al.*, 1997; Heal *et al.*, 1998*b*) would argue against circadian variation in stored 5-HT levels making a sizable impact on fenfluramine efficacy. So to would the reasoning of Curzon *et al.* (1997), who describe that 5-HT receptors are required for appetite suppression but high levels of 5-HT itself are not. Yet in the clinic fenfluramine does vary in efficacy across the circadian cycle (at least in concert with phentermine; Katz *et al.*, 1999), and indeed in the work on the BSS described above [chapter 7, and to a lesser degree chapter 5]; racemic fenfluramine also exhibits rhythmicity when looking strictly at food intake in animals (Davies and Wellman, 1991), although this particular finding was not supported by the present work. Sibutramine exerts actions over both 5-HT and NA. The satiety enhancing function of sibutramine, and its promotion of thermogenesis, are both dependent on the inhibition of re-uptake of both neurotransmitters (*e.g.* Heal *et al.*, 1998*a*). The synergistic effect of inhibiting re-uptake of the two neurotransmitters is such that one might expect the potency to exhibit some level of dependence on the circadian rhythmicity of either NA or 5-HT systems, or both. Furthermore, since reuptake inhibition requires neuronal firing to raise neurotransmitter levels (*e.g.* Gundlah *et al.*, 1997) one might expect the efficacy of treatment with re-uptake inhibitors to be closely tied to circadian variation in neuronal firing rates. These factors suggest that it would be interesting to study the interplay between the two transmitters over the circadian cycle. Specifically, it would be pertinent to examine any circadian variation in the relative levels of 5-HT and NA, and in the degree of change induced after dosing with sibutramine - especially in regions of the hypothalamus like the PVN where both these neurotransmitters have a prominent role in the control of feeding (*e.g.* Leibowitz and Alexander, 1998; Leibowitz and Hoebel, 1998; Wellman *et al.*, 1993). Such studies would begin to illuminate the correlation between the neurochemical and behavioural responses that

eliciting further understanding of how multi-substrate drug treatment of obesity may be targeted for maximal effect.

Further work may also be appropriate to examine the effect of the drugs on macronutrient selection and whether this impact varies over the circadian cycle. Interactions between food choices and calorific load required to satiate may mean that the ideal dosing schedules are not those which simply aim to satiate soonest, if the aim is to maintain a balanced diet. Furthermore, modulations of serotonin are associated with effects on macronutrient intake (*e.g.* Leibowitz and Alexander, 1998; Leibowitz and Hoebel, 1998), with a selective suppression of carbohydrate intake reported to be associated with hypothalamic increases in 5-HT (*e.g.* Leibowitz and Alexander, 1998; Leibowitz and Shor-Posner, 1986). The degree of this selectivity and the efficacy of the inhibition are just two factors which may differ with circadian time.

Circadian variation in the pharmacokinetics of sibutramine, and to a lesser extent *d*-fenfluramine, would also be worth investigating. Both drugs have active metabolites and in the case of sibutramine the metabolites are more relevant to the clinical action than the parent compound (*e.g.* Heal *et al.*, 1998*a*; Rowland and Carlton, 1986; Simansky, 1996). The efficiency with which they undergo metabolism – both from parent compound to active metabolites and the clearance of these metabolites – is clearly a relevant issue when looking at drug efficacy. Given that circadian changes in metabolism and other pharmacokinetic measures are well characterised with a number of drugs (*e.g.* Labreque and Bélanger, 1991), there is every reason to suspect that such variation could be mirrored in the case of sibutramine or fenfluramine. Likewise the binding profiles of the drugs may change with circadian time, especially if the level of 5-HT re-uptake carrier expression varies, and consequently influences drug efficacy. This is especially true in the case of fenfluramine, with which, in addition to requiring the carrier to enter neurons and cause neurotransmitter release, there is a suspicion that a direct action on 5-HT receptors by the parent compound may contribute to the clinical effect (Curzon *et al.*, 1997). Furthermore norfenfluramine, a fenfluramine metabolite, is accepted as having direct agonist potency at 5-HT_{2C} receptors (Gibson *et al.*, 1993; Simansky, 1996) and so circadian variation in the function or expression of this receptor could potentially affect the efficacy of *d*-fenfluramine treatment.

Similarly it is thought that 5-HT acts through 5-HT_{1B} receptors to enhance satiety (*e.g.* Blundell and Halford, 1999; Simansky, 1996) and this receptor has been shown to exhibit functional variation with circadian time (Garabette *et al.*, 2000), at least in the SCN which is tangentially associated with feeding (*e.g.* Leibowitz and Alexander, 1998).

Sibutramine also enhances thermogenesis (Connoley *et al.*, 1999; Heal *et al.*, 1998*a*; Stock, 1997). It is entirely possible that the metabolic response to sibutramine also varies with circadian time and since the neuronal circuits involved are different (though based on the same neurotransmitters) the pattern of circadian variation could well be different. When the drug is most effective at stimulating thermogenesis is a moot point when strictly looking at the efficacy to enhance satiety or reduce food intake, but it is entirely relevant to the overall efficacy of the drug. Study of circadian variation in the thermogenic potential of sibutramine would be essential to selecting a dosage regime for maximal efficacy. It would be of fundamental interest to see how the rhythmic changes in the efficacy of sibutramine match up on either side of the energy balance.

Another key point to consider is that 5-HT and NA are far from the only endogenous modulators of food intake and feeding behaviour. Another monoamine – dopamine – has a role and there are extensive implications for a variety of neuronal and hormonal peptides (*e.g.* Leibowitz and Hoebel, 1998; Wynne *et al.*, 2005). The likelihood is that some, if not most or even all, of these modulators exhibit some level of circadian variation. This could be down to a number of factors, including cycles in synthesis, release or metabolism. Whilst the circadian variation in 5-HT and NA is likely to be of more relevance to the changes in response to anorectics that exert their primary effects on these systems, changes in the associated chemical backdrop are possibly a factor and should perhaps be investigated – if not for their relevance to sibutramine or *d*-fenfluramine anorexia then in relation to drugs that work through those systems.

Hand in hand with this is the idea that treatments which act to enhance satiety by their very nature require a certain level of feeding to show an effect. It also follows that if one is looking to inhibit food intake generally then treatment is more likely to be effective when given during periods when feeding is naturally high (*e.g.* Davies and

Wellman, 1991). This likelihood grows if the primary mechanism of the drugs used is to facilitate and enhance the natural process of meal termination – satiation. The current work was not primarily concerned with food intake *per se*, although reducing food intake is inextricably linked to anti-obesity therapy and the enhancement of satiety. The aim of the work presented here was more focused on trying to determine whether two anti-obesity agents, sibutramine and *d*-fenfluramine, exhibited circadian variation specifically in their activity to enhance satiety – hence the use of the BSS, and a time sampling mechanism, as opposed to a paradigm tailored to specific measurement of food intake and detailed meal patterning. Naturally the current approach would have been more valuable had it proved possible to carry out the corresponding dialysis studies alongside the BSS observations. Such a pairing would have allowed an estimate of pharmacological efficacy at the neurochemical level, as well as at the behavioural level.

In hindsight, having been unable to perform both the behavioural and neurochemical experiments, the BSS experiments described in chapter 7 would perhaps have been better carried out more stringently, using continuous analysis. This would have allowed more detailed dissection of the time-courses involved and the nature of the expression of the satiety sequence across the 24 hour day. However, as mentioned in the discussion of why the time-sampling method used was chosen [section 4.4], continuous analysis would have been a lot more time intensive to set up and run and this would have had a negative impact on the attempts then in motion to set up a microdialysis program alongside the behavioural work. Ultimately this program failed to reach fruition in any case, and looking back it was perhaps a mistake to attempt to give full attention to both the behavioural and the neurochemical to such a degree.

This aside, what was learnt from the experiments presented above?

First, in agreement with previous literature (Rowley *et al.*, 2000; Vickers *et al.*, 2000) significant locomotor retardation or sedation was not an issue with the doses used for circadian work, with neither sibutramine nor *d*-fenfluramine inducing a significant decrease in exploratory locomotor paradigms [chapter 6]. This is not to say that neither drug affected the level of behavioural activity however, as both were seen to have effects on activity counts in the BSS to different degrees and at different times of

day (**figure 7.5.19**, **table 7.5.1**). It is also worth noting that whilst it was *d*-fenfluramine that proved the more effective modulator of activity counts in the BSS (**table 7.5.1**) it was sibutramine that had the greater impact on locomotor activity when that was quantified [chapter 5]. The pattern of that locomotor impact was consistent with the animals reaching a state of post-ingestive satiety, and the associated resting state, sooner; the initial levels of activity tended to match that seen after saline, but the animals reached a plateau in locomotor performance earlier. This finding was entirely consistent with the observations that, as in these studies [chapters 4 and 7] and in previous reports (Halford *et al.*, 1995, 1998; Heal *et al.*, 1998*a*), sibutramine advanced and preserved the BSS. Similarly *d*-fenfluramine advanced the BSS [chapters 4 and 7], consistent with the accepted effect (Halford *et al.*, 1995, 1998). However the pattern seen in locomotor activity studies at CT5, mid light phase, was for *d*-fenfluramine treatment to inhibit locomotor activity early in the recording period but for a plateau phase not to be reached as it was under sibutramine and saline conditions at that circadian time. At CT5 animals showed more locomotion in the latter half of the recording after receiving *d*-fenfluramine than after receiving saline. This finding is in agreement with earlier reports that fenfluramine disrupted the satiety sequence by interfering with resting behaviour (Montgomery and Willner, 1988; Willner *et al.*, 1990; McGuirk *et al.*, 1992*a*), a view that is not generally supported (Halford *et al.*, 1998) and is not in agreement with the BSS results presented above [chapters 4 and 7]. There are signs from BSS observations at CT5 (**figures 7.5.4-7.5.6**) that *d*-fenfluramine treatment advanced the BSS, although there was a "second meal" and a fall in resting counts after the half-way point of the observations. This could perhaps be interpreted as a disruption of the satiety sequence, but one could equally argue that the sequence had by then already been expressed. Furthermore, 5-HT is considered to be a short-term satiety factor rather than a long term signal (*e.g.* Leibowitz and Hoebel, 1998) so far from being inconsistent with *d*-fenfluramine causing an advance in the BSS the present findings support the idea that *d*-fenfluramine advances the BSS. Despite this, however, the locomotor activity profile produced with *d*-fenfluramine at CT5 (and such a profile was only seen at CT5) could suggest a reason why other researchers observed a disruption of the BSS with acute fenfluramine since the studies suggesting fenfluramine interfered with resting were carried out in the mid-light phase (Montgomery and Willner, 1988; Willner *et al.*, 1990; McGuirk *et al.*, 1992*a*).

As to what might be behind this response, the current findings give no insight. It was only seen at CT5, so either there was a factor specific to this time that contributed to the findings or the results represent an anomaly. The latter seems unlikely given the correlation with previous observations with fenfluramine when used at equivalent circadian times (Montgomery and Willner, 1988; Willner *et al.*, 1990; McGuirk *et al.*, 1992*a*). On the other hand there is good evidence that the response to drugs is susceptible to circadian variation. Mid light phase is a time of peak tissue 5-HT concentration (*e.g.* Martin and Redfern, 1997), and so the pool of transmitter released may be significantly higher at this time than at others; since 5-HT activity is linked to general behavioural arousal it is plausible that the drug is having wider reaching effects than the specific satiety response seen at other times. This could be because the activating effect of the serotonergic response is more than compensating for the non-serotonergic sedative effect; Callaway *et al.* (1993) showed that fenfluramine produced its sedative effects – normally seen only at higher concentrations – via a non-serotonergic mechanism. There is also the possibility that the weak NA releasing potential of fenfluramine (Rowland and Carlton, 1986) is playing a role – with NA again implicated in general arousal. One hypothesis consistent with the results shown here is that the specific satiating effect is seen initially, but the short-lasting 5-HT satiety signal wears off and the resting associated with satiety is then reduced by 5-HT-driven behavioural activation.

That it is not clear how or why this effect is mediated but that it is none the less there goes hand in hand with a basic premise of this thesis: that circadian rhythm is a significant modulating factor and that effects caused by circadian variation ought to be substantiated and controlled for. Ideally, for complete understanding, the effect of individual drugs on the BSS should be assessed across the light-dark cycle, but this is unlikely to be achieved. It has long been argued that such behavioural anorectic studies are best carried out in the phase of natural activity (*e.g.* Halford *et al.*, 1998) yet the current study is the first to systematically compare the effects of two anorectics across the full circadian cycle in a paradigm measuring behavioural changes in addition to food intake. The ability of some drugs to affect the extent of food intake had been subject to tests across the circadian cycle before (Davies and Wellman, 1991) but this measure is heavily dependent on the base level of intake

under control conditions and potentially not illustrative of the behaviour and/or the mechanisms behind the reduction. The BSS experiments described above [chapter 7] found that the sequence was preserved across all circadian times at which a significant feeding response was seen, and moreover was advanced by the satiety enhancing agents, sibutramine and *d*-fenfluramine, at each time except late in the light phase (CT9) when the sequence was not present under control conditions either.

The degree to which the drugs were effective in advancing the satiety sequence was subject to slight variation – as determined by when the effect on certain behaviours after drug treatment became significantly different from controls (*e.g.* **table 7.5.1**) – but the more pertinent finding was that treatment with either drug muted changes seen between the groups of animals across the circadian cycle when comparing only matching drug treatments from each circadian time. The same differences in the offset of feeding and onset of resting that were observed at different times of day in saline treated animals were not universally preserved in animals treated with either drug. Unsurprisingly the drugs appeared to have been most effective in the dark phase, when feeding was at its peak, with the rhythmic differences between dark- and light-phase feeding and resting largely obscured by drug treatment. Effectively giving either drug in the dark phase caused the animal to behave in manner more like that shown in the light phase – eating less, satiating sooner, and resting more. This is unsurprising; both drugs are satiety enhancers and they work to accelerate the natural process of meal termination. If the basic meal size is larger then the chances are it will naturally last longer, so the potential for reduction in time spent feeding is greater. On the other hand, arguably the most classical and clear advancements of the satiety sequence – to judge solely on the behavioural profiles – were observed at CT5 (mid light phase, when sibutramine advanced the BSS but did not significantly reduce food intake relative to saline and when *d*-fenfluramine appeared to prevent long periods of rest given locomotor results) and at CT21 late in the dark phase.

Ultimately the clarity of shift as shown in the behavioural profile means little; there is no question, from these results, that either sibutramine or *d*-fenfluramine exerted their anorectic effect by anything other than satiety enhancement at any time, when given a dose appropriate to their action to inhibit food intake. Higher doses of fenfluramine would likely demonstrate a degree of sedation in the response which

may or may not vary with circadian time; the sedative potential of higher doses of *d*-fenfluramine was illustrated in the exploratory locomotor activity study described above [chapter 6] and the pre-existing body of work describing fenfluramine inhibition of motor activity (Aulakh *et al.*, 1988; Callaway *et al.*, 1993, Ziance *et al.*, 1972). Sibutramine is unlikely to be sedative at any time, again in agreement with previous work (Rowley *et al.*, 2000). The locomotor profiles [chapter 5] by and large agree with the BSS findings [chapter 7], and circadian-independent reduction in locomotor activity in sibutramine treated animals is, far from being indicative of sedation, consistent with an earlier onset of satiety-induced post-prandial resting. There is a slight suggestion that the sedative potential of fenfluramine may have been a significant factor at CT17, where locomotor activity significantly differed from the saline condition, but this was not evident as a disruption of the BSS at this, or any other, circadian time.

9.0 Summary

In summary the present work supports the notion that both sibutramine and *d*-fenfluramine are anorectic drugs that produce their hypophagic effect through the enhancement of post-ingestive satiety – accelerating natural meal termination. This is true regardless of when in the 24 hour day the drug is given. Other mechanisms such as hyperactivity or sedation, the latter particularly a possibility with *d*-fenfluramine, do not appear to play any significant role at any time, at the doses used here. There is a suggestion that *d*-fenfluramine may have slightly differential effects on motor activity at mid-light or mid-dark which could potentially explain some previous discrepancies with this drug in the BSS (Montgomery and Willner, 1988; Willner *et al.*, 1990; McGuirk *et al.*, 1992*a*). Unsurprisingly both sibutramine and *d*-fenfluramine appear more potent at enhancing satiety, if not inhibiting food intake *per se*, during the dark phase when natural feeding is more prevalent, and targeting drug administration to times of peak food intake, *i.e.* before main meals, would appear to be a successful strategy to maximise the satiating effect.

10.0 References

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11.0 Appendix

11.1 The following pieces of software were used in the compilation of this thesis:

Microsoft Word 2002 – to write and compile the text and integrate the figures.

Microsoft Excel 2002 – to record and work with raw data and produce and customise figures.

MDL ISIS/Draw – to draw and show the chemical structures.

StatView for Windows 5.0.1 (SaS Institute Inc.) and SPSS 12.0 were used for statistical analyses.

The locomotor activity data was collected with AM Logger v. 1.216 from CNT limited.

11.2 Sample data sheets used for recording behaviours during BSS observations, shown smaller than actual size.

Observation no.	Rat 1	Rat 2	Rat 3
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Observation no.	Rat 4	Rat 5	Rat 6
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Observation no.	Rat 1	Rat 2	Rat 3
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Observation no.	Rat 4	Rat 5	Rat 6
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11.3 Sample dosing schedule; indicated is the random order of dosing for each animal under paradigms where animals were tested with more than one treatment. This example was used for the experiments carried out at CT5.

		Run	
Animal	1	2	3
1	d-fen	**Sal**	**1.67 sib**
2	d-fen	**1.67 sib**	**Sal**
3	**Sal**	**1.67 sib**	d-fen
4	**1.67 sib**	d-fen	**Sal**
5	**Sal**	d-fen	**1.67 sib**
6	**1.67 sib**	**Sal**	d-fen
7	**Sal**	**1.67 sib**	d-fen
8	**1.67 sib**	**Sal**	d-fen
9	**Sal**	d-fen	**1.67 sib**
10	d-fen	**1.67 sib**	**Sal**
11	**1.67 sib**	d-fen	**Sal**
12	d-fen	**Sal**	**1.67 sib**